Year : 2021

Insect eggs induce a systemic acquired resistance against Botrytis cinerea

Alfonso Esteban

Alfonso Esteban, 2021, Insect eggs induce a systemic acquired resistance against Botrytis cinerea

Originally published at : Thesis, University of Lausanne

Posted at the University of Lausanne Open Archive http://serval.unil.ch
Document URN : urn:nbn:ch:serval-BIB_73FFDE94D9D81

UNIL | Université de Lausanne
Faculté de biologie
et de médecine

Département de Biologie Moléculaire Végétale (DBMV)

Insect eggs induce a systemic acquired resistance against *Botrytis cinerea*

Thèse de doctorat ès sciences de la vie (PhD)

présentée à la

Faculté de biologie et de médecine
de l'Université de Lausanne

par

Esteban ALFONSO

Master en Sciences Moléculaires du Vivant de l'Université de Lausanne

Jury

Prof. Paul Franken, Président
Prof. Philippe Reymond, Directeur de thèse
Prof. Anke Steppuhn, Experte
Prof. Brigitte Mauch-Mani, Experte

Lausanne, 2021

UNIL | Université de Lausanne
Faculté de biologie et de médecine

Ecole Doctorale
Doctorat ès sciences de la vie

Imprimatur

Vu le rapport présenté par le jury d'examen, composé de

Président·e	Monsieur	Prof.	Paul	**Franken**
Directeur·trice de thèse	Monsieur	Prof.	Philippe	**Reymond**
Expert·e·s	Madame	Prof.	Anke	**Steppuhn**
	Madame	Prof.	Brigitte	**Mauch-Mani**

le Conseil de Faculté autorise l'impression de la thèse de

Monsieur Esteban Alfonso

Maîtrise universitaire ès Sciences en sciences moléculaires du vivant, Université de Lausanne

intitulée

Insect eggs induce a systemic acquired resistance against *Botrytis cinerea*

Date de l'examen : 17 août 2021

Date d'émission de l'imprimatur : Lausanne, le 24 août 2021

pour le Doyen
de la Faculté de biologie et de médecine

Prof. Niko GELDNER
Directeur de l'Ecole Doctorale

CONTENT

SUMMARY

Despite their inert stage, insect eggs deposited on leaves represent a considerable threat since they will develop into feeding larvae. Plants respond actively to insect eggs deposition by triggering various defences that lead to egg desiccation, drop-off, mortality, or attraction of egg parasitoids. Upon *Pieris brassicae* oviposition, *Arabidopsis thaliana* activates the salicylic acid (SA) pathway, which is usually induced to fend off bacterial pathogens. Recently, it was shown that *P. brassicae* oviposition and treatment with egg extract (EE) induce a systemic acquired resistance (SAR) against the bacterial pathogen *Pseudomonas syringae* in *A. thaliana*. The aim of this thesis was to investigate whether egg-induced SAR was also effective against the fungal necrotroph *Botrytis cinerea*.

Here, we show that *P. brassicae* oviposition and EE treatment induce SAR against *B. cinerea*. This effect was reproducible when plants were treated with EE from the generalist *Spodoptera littoralis*. Furthermore, pretreatment with a solution of phosphatidylcholines, which are active compounds found in *P. brassicae* and *S. littoralis* eggs, reduced *B. cinerea* growth to the same extent as EE. This indicates that egg-induced SAR is triggered following perception of an egg-associated molecular pattern. EE-induced SAR is abolished in *ics1*, *ald1* and *fmo1* mutants, indicating that the SA and *N*-hydroxy-pipecolic acid pathways are involved. In addition, we found that EE-induced SAR establishment requires tryptophan-derived metabolites, with a substantial contribution of camalexin, a known antifungal compound. Indeed, we found that SAR is abolished in several mutants deficient in camalexin biosynthesis, including *cyp79b2 cyp79b3*, *cyp71a12 cyp71a13*, *pad3-1* and *wrky33*. Although we found that expression of the camalexin biosynthesis gene *PAD3* is primed by EE treatment, metabolic analyses revealed that levels of camalexin are not different between control and EE-treated plants, leaving open the question on how camalexin exerts its action. Finally, we found that EE treatment also reduced infection by an oomycete pathogen, illustrating the broad range activity of EE-induced SAR.

We also demonstrate that *P. brassicae* larvae performance is reduced in *B. cinerea*-infected plants, which implies that egg-induced SAR might benefit the insect. This phenomenon might illustrate a strategy of the insect that manipulates plant SA pathway through oviposition to reduce pathogen infections that are detrimental for future hatching larvae.

RÉSUMÉ

Bien qu'ils constituent un stade inerte de leur vie d'insecte, les œufs représentent en réalité une véritable menace pour la plante. En effet, ceux-ci vont finir par éclore et les chenilles en résultant vont se nourrir sur les feuilles, causant des dommages importants. Les plantes ont développé plusieurs défenses en réponse aux œufs, qui vont amener au desséchement, à la chute ou à la mort de ces derniers. La plante émet également des composés volatiles attirant des guêpes parasitoïdes, qui vont attaquer les œufs. En réponse aux œufs de la piéride du chou *Pieris brassicae*, la plante modèle *Arabidopsis thaliana* active des défenses liées à la voie de l'acide salicylique, une voie habituellement induite en réponse à des infections microbiennes. Récemment, il a été montré que les œufs de *P. brassicae* induisent une résistance systémique acquise (SAR) contre le pathogène bactérien *Pseudomonas syringae*. L'objectif de cette thèse était de vérifier si cet effet s'applique aussi au pathogène fongique *Botrytis cinerea* et d'en caractériser les mécanismes moléculaires.

Nous montrons que les œufs de *P. brassicae* induisent une SAR contre *B. cinerea*. De plus, l'application de phosphatidylcholines, des phospholipides contenus dans les œufs d'insectes, induit une SAR contre *B. cinerea*, de manière similaire aux œufs. Ceci indique que la SAR induite par les œufs est déclenchée à la suite de la perception de signatures moléculaires associées aux œufs. La SAR nécessite l'accumulation d'acide salicylique et d'acide hydroxy-pipécolique, deux molécules indispensables à la génération de cette réponse. De plus, nous montrons que l'induction de la SAR requiert des composants du métabolisme de l'indole avec une contribution majeure de la camalexine, un composé antifongique. En effet, nous constatons que plusieurs mutants bloqués dans la synthèse de la camalexine n'activent pas de SAR. Comparé à des plantes non traitées, le traitement aux œufs induit une expression plus rapide et plus forte de *PAD3*, un gène de biosynthèse de la camalexine. Cependant, les quantifications de ce métabolite ne montrent pas de différences entre plantes infectées par *B. cinerea* précédemment traitées aux œufs ou non, posant la question du mode d'action de la camalexine. Finalement, nous montrons que le traitement aux œufs réduit aussi l'infection causée par un oomycète, montrant que la SAR induite par les œufs agit à large spectre.

Nous démontrons également que les larves de *P. brassicae* se développent moins bien sur des plantes infectées par *B. cinerea*, ce qui suggère que la protection induite par les œufs serait bénéfique pour l'insecte. Ce phénomène unique pourrait constituer une stratégie de l'insecte qui manipulerait, par la ponte de ses œufs, l'induction de défenses afin de réduire l'effet néfaste des pathogènes, au bénéfice de sa progéniture.

GENERAL INTRODUCTION

Life on Earth would not exist without the existence of plants. Plants are the main primary producers and constitute the basis of the world's food chain. Being photo-autotrophic organisms, plants convert energy from sunlight into chemical energy stored in sugars, and release oxygen in the atmosphere as a waste product, a process called photosynthesis. Heterotrophic organisms, that cannot produce their own food, use both of these photosynthesis products to generate energy via cellular respiration and thus fuel their metabolic activities. Beside this major ecological role, plants are incredibly useful to humans by providing essential materials such as wood to build tools and housing or fiber to make clothes and paper. Plants also produce a huge variety of molecules, some of which are used nowadays as drugs in modern medicine, others can be a source of biofuels and biodegradable resources.

Modern industrial societies are responsible for the fast growth rate of human population and one of the constant challenges of plant science is to enhance food production in order to sustain the needs. Nowadays, large monoculture crop fields are covering lands and often replacing natural habitats, a phenomenon called "landscape simplification" (Thies et al., 2003). However, landscape simplification is thought to increase pest pressure on crops by facilitating their establishment and by reducing the abundance of pest natural enemies, ultimately leading to reduced yields and increased use of pesticides (Meehan et al., 2011; Gagic et al., 2021). With an estimated 30% of crop losses due to pests and pathogens (Douglas, 2018), which is likely to increase due to global warming (Deutsch et al., 2018), it is of great importance to study plant immunity and plant-insect/pathogen interactions in order to develop new strategies for better economic and environmental outcomes.

Plant immunity

Plants are constantly exposed to a wide variety of biotic stresses including phytopathogens such as bacteria, fungi, viruses and oomycetes but also herbivory. Plants have co-evolved for millions of years with these invaders, thus developing specific protective mechanisms against them. In turn, these attackers have evolved ways to circumvent plant defences, shaping plant immunity in a constant arms race. Constitutive physical and chemical defences assure a first barrier. Typical physical barriers include plant cell wall, cuticle and thorns, that will help to prevent entry of pathogens or repel herbivores. However, several pathogens are able to break

through, mainly by deploying degrading enzymes. Plant chemicals can be divided in two classes, the primary metabolites, which consist of molecules indispensable for growth and development such as sugars, amino acids, proteins and secondary metabolites, which are used as defence compounds (Piasecka et al., 2015). Secondary metabolites can be further divided in two classes according to their mode of biosynthesis and action. Phytoanticipins are produced and stored in a constitutive manner and deployed only in case of attack, such as saponins, glucosinolates and cyanogenic glucosides, whereas phytoalexins are synthesized in response to an infection, such as the *Brassicaceae*-specific camalexin and terpenoids.

The second line of plant defences is inducible, activated upon the recognition of microbial and herbivore-derived signature molecules, termed as pathogen-associated molecular patterns (PAMPs) or herbivore-associated molecular patterns (HAMPs), respectively. PAMPs are highly conserved and essential compounds, such as the 22 amino acids peptide derived from bacterial flagellin (flg22), bacterial/fungal cell wall and membrane compounds like peptidoglycan, chitin and lipopolysaccharide (LPS) as well as many others (Ranf, 2017; Yu et al., 2017). PAMPs recognition by plasma membrane-localized pattern recognition receptors (PRRs) triggers the activation of a specialized immune response known as pattern-triggered immunity (PTI) (Jones and Dangl, 2006). Two types of PRRs have been described, receptor-like kinases (RLKs), which contain an extra-cellular domain, a trans-membrane domain and a cytoplasmic kinase domain, and receptor-like proteins (RLPs) lacking the cytoplasmic kinase domain (Couto and Zipfel, 2016). Most of the PRRs associate with co-receptors, such as BAK1, to transduce downstream PTI responses (Roux et al., 2011). Following PRR/co-receptor complex formation, a series of signalling events rapidly takes place and this involves an increase of intracellular Ca^{2+} levels, activation of mitogen-activated protein kinases (MPK) regulating defence gene expression, generation of a reactive oxygen species (ROS) burst, accumulation of defence hormones leading to large transcriptional changes to finally produce defence metabolites and proteins (Yu et al., 2017; Zhou and Zhang, 2020). However, a lot of pathogens manage to circumvent plant defences by delivering effector proteins that can suppress PTI responses at different levels. In response to this adaptation, plants evolved a way to directly or indirectly detect the presence of effectors with resistance proteins (R proteins), leading to a stronger immune response called effector-triggered immunity (ETI) (Jones and Dangl, 2006). ETI responses lead to the generation of a localized cell death called hypersensitive response (HR), ROS burst, accumulation of defence hormones and pathogenesis-related (PR) proteins (Yu et al., 2017; Bürger and Chory, 2019). However, recent reports indicate that PTI and ETI signalling are actually intricately linked since both PRRs and

R proteins are required to mutually potentiate strong immune responses in *Arabidopsis thaliana* (hereafter *Arabidopsis*), bringing new understanding of plant immunity (Ngou et al., 2021; Yuan et al., 2021).

Although much less information is available on the recognition of HAMPs and downstream signalling steps, as well as for the presence of insect-derived effectors, evidence is accumulating that similar molecular mechanisms are used to detect and respond to microbes and arthropods (Stahl et al., 2018; Erb and Reymond, 2019; Arimura, 2021).

Plant defences are orchestrated mostly by three hormones: salicylic acid (SA), jasmonic acid (JA) and ethylene (ET) (Bari and Jones, 2009). Different infection strategies exist according to pest/pathogen lifestyles and plants have to respond consequently. Phytopathogens can be classified in two main groups: biotrophs and necrotrophs. Biotrophs keep their hosts alive to feed on living tissues, whereas necrotrophs kill their hosts cells before obtaining nutrients from them. Some pathogens are hemi-biotrophs, starting with a biotrophic lifestyle and switching to a necrotrophic phase later in the infection process. Results from several studies have shown that plants activate SA-dependent defence responses against biotrophic pathogens (Glazebrook, 2005; Vlot et al., 2009). Plants use two different routes to synthesize SA, the isochorismate synthase (ICS) and the phenylalanine ammonia-lyase (PAL) pathways (Peng et al., 2021). Upon pathogen infection, most of the SA is produced via the ICS pathway, which starts from chorismate (Wildermuth et al., 2001) (Fig. 1A). *Arabidopsis* contains two genes encoding isochorismate synthases, *ICS1* and *ICS2*, but only *ICS1* is expressed upon pathogen infection (Wildermuth et al., 2001). Consequently, *ics1* single mutant (also known as *salicylic-induced deficient 2, sid2*), is unable to accumulate high levels of SA and is blocked in SA-dependent responses, whereas *ics2* still exhibits wild-type SA levels (Nawrath and Métraux, 1999; Garcion et al., 2008). However, the *ics1 ics2* double mutant accumulates even less SA than *ics1* single mutant in response to UV stress, suggesting a minor contribution of ICS2, highlighting the unequal redundancy of these two enzymes (Garcion et al., 2008). Once produced, isochorismate can be transported out of the chloroplast via the transporter ENHANCED DISEASE SUSCEPTIBILITY 5 (EDS5), where the cytosolic amidotransferase AvrPphB SUSCEPTIBLE 3 (PBS3) conjugates it to glutamate to generate isochorismate-9-glutamate, an unstable compound that can spontaneously decay into SA or be converted to SA even faster by ENHANCED PSEUDOMONAS SUSCEPTIBILITY 1 (EPS1) (Rekhter et al., 2019; Torrens-Spence et al., 2019). To regulate defence gene expression, SA is then perceived by two classes of receptors in the nucleus, NON-EXPRESSOR OF PATHOGENESIS-RELATED GENES 1 (NPR1) and NPR1-LIKE PROTEIN 3/4 (NPR3/NPR4) (Fu et al., 2012).

NPR proteins directly interacts with TGACG MOTIF-BINDING FACTOR (TGA) transcription factors TGA2/TGA5/TGA6 to modulate gene expression (Peng et al., 2021). NPR1 has been shown to be a transcriptional activator of SA responses whereas NPR3/4 act as transcriptional repressors. SA binding to NPR1 enhances its transcriptional activator activity and inhibits the transcriptional repression of NPR3/4, leading to induction of defence genes (Ding et al., 2018; Peng et al., 2021). Furthermore, a recent report indicates that both activation of NPR1 and repression of NPR3/4 by SA are required for PTI, ETI and systemic defence responses (Liu et al., 2020). SA-dependent responses mainly consist in local containment of disease progression and protection of systemic tissues against further infections. This is achieved by the transcription of *PR* genes whose products are known to have antimicrobial properties deterring bacterial, fungal and viral infections (Sels et al., 2008). On the other hand, plant defences against necrotrophic pathogens and herbivores largely depend on JA signalling pathway, which is known to be antagonistic to SA (Reymond et al., 2004; Pieterse et al., 2009). Indeed, SA-dependent cell death induction does not protect plants against necrotrophs but rather promotes susceptibility to them (Govrin and Levine, 2000; Glazebrook, 2005). JA biosynthesis initiates in the chloroplast with the oxygenation of linolenic acid by lipoxygenases (LOX) enzymes to generate 12-oxo-phytodienoic acid (OPDA), which is converted to JA and further conjugated to isoleucine (Ile) by JASMONATE-RESISTANT 1 (JAR1) to generate the bioactive form, JA-Ile (Howe and Jander, 2008; Fonseca et al., 2009). In the absence of JA-Ile (plant resting state), negative regulators belonging to the family of JASMONATE ZIM DOMAIN (JAZ) proteins repress JA-dependent transcription factors, such as MYC2, MYC3 and MYC4, by recruiting the general corepressor TOPLESS (TPL) through an interaction with the adaptor protein NOVEL INTERACTOR OF JAZ (NINJA) (Pauwels et al., 2010). When the plant is damaged (upon herbivory or necrotrophic infection), JA-Ile accumulates rapidly and promotes the binding of JAZ to CORONATINE INSENSITIVE 1 (COI1), an F-box protein that is part of the Skp1/Cullin/F-box SCF^{COI1} ubiquitin E3 ligase complex, mediating ubiquitination of JAZs and targeting them for proteasome degradation (Chini et al., 2007; Thines et al., 2007). Once JAZs are degraded, MYC2/3/4 are released from repression and initiate the transcription of JA-responsive genes. MYC2/3/4 regulate the biosynthesis of glucosinolates, a class of defence compounds specific to the *Brassicaceae* family and important to fend off chewing herbivores. Accordingly, the triple mutant *myc2 myc3 myc4* is devoid of glucosinolates and is highly susceptible to feeding by the generalist *Spodoptera littoralis* (Schweizer et al., 2013). Furthermore, glucosinolates breakdown products have been shown to be detrimental to several fungal pathogens (Bednarek et al., 2009). ET is a methionine-derived

hydrocarbon gas, which coupled to JA, induces another branch of the JA pathway, regulating signalling components such as ETHYLENE RESPONSE FACTOR (ERF) transcription factors to regulate several genes such as *PLANT DEFENSIN 1.2* (*PDF1.2*), which is important for defence against necrotrophic pathogens (Bürger and Chory, 2019).

Plant responses to insect eggs

After mating, female butterflies have a challenging task, which is to deposit eggs onto a suitable host plant, to ensure their progeny's survival. Ovipositing insects mainly rely on external stimuli to recognize their hosts, such as visual and olfactory cues. Specialist lepidopteran insects are known to select their host plants through the detection of host-specific chemicals. The crucifer specialist *Plutella xylostella* uses intact glucosinolates as recognition cues for oviposition (Sun et al., 2009). The monarch butterfly *Danaus plexippus*, a milkweed specialist, uses specialized chemoreceptors to sense leaves flavonoids and thus detect its hosts for oviposition (Haribal and Renwick, 1996). Despite their inert stage, insect eggs represent a considerable threat since they will develop into feeding larvae, causing important damages. Plants respond actively to insect eggs deposition by triggering various direct and indirect defences.

Direct defences aim to target directly the eggs and not the ovipositing female and can have various outcomes such as eggs desiccation, detachment, destruction and killing (Reymond, 2013; Hilker and Fatouros, 2015; Bertea et al., 2020). Following oviposition, plants induce a localized ROS burst, callose deposition and cell death, leading to eggs desiccation or falling. This reaction is described as a hypersensitive-like response (HR-like) due to the intriguing similarities shared with pathogen-triggered HR (Reymond, 2013). This response was described in several species of *Brassicaceae* and *Solanaceae* families (Shapiro and DeVay, 1987; Balbyshev and Lorenzen, 1997; Fatouros et al., 2012; Bonnet et al., 2017; Griese et al., 2021). Other plants grow tumour-like structures called neoplasm underneath the eggs, leading to their detachment and increasing egg/larval mortality (Doss et al., 2000; Petzold-Maxwell et al., 2011; Geuss et al., 2017). Moreover, some plants developed ovicidal substances, such as rice varieties that produce benzyl benzoate to kill eggs of several planthoppers species (Seino et al., 1996) or the bittersweet night-shade *Solanum dulcamara* accumulating toxic amount of hydrogen peroxide (H_2O_2) at the site of oviposition (Geuss et al., 2017). Another fascinating example of a direct egg defence is in *Viburnum* spp. where eggs from the leaf beetle *Pyrrhalta*

viburni are deposited in cavities they dig in twigs. By growing wound tissue in response to oviposition, eggs are crushed in the cavity before even hatching (Desurmont et al., 2011).

Indirect defences, on the contrary, aim to indirectly harm the eggs, by attracting natural enemies. Oviposition-induced plant volatiles (OIPVs) emitted in the air attract parasitoid wasps that exclusively attack and kill the deposited eggs and not the host plant. It has been demonstrated that oviposition by the elm leaf beetle *Xanthogaleruca luteola* and the pine sawfly *Diprion pini* induces the emission of terpenoid volatiles that specifically attract female egg parasitoids (Wegener et al., 2001; Hilker et al., 2002; Mumm et al., 2003; Büchel et al., 2011). Interestingly, even some insectivorous birds can scent and be attracted by OIPVs (Mäntylä et al., 2018; Mrazova et al., 2019). In addition to OIPVs, eggs also induce indirect defences by triggering changes in the plant's surface chemistry. Parasitoids perceive these changes while walking on the plant and spend more time on it, thus increasing chances to find and parasite eggs (Fatouros et al., 2005; Fatouros et al., 2008). Finally, indirect defences can also result from morphological rather than chemical innovations. Plants of the *Passiflora* genus contain extensive chemical defences such as various cyanogenic glucosides, flavonoids and alkaloids, but specialist insects such as *Heliconius* spp. can handle them. To circumvent that adaptation, some *Passiflora* species have evolved egg-like structures dispersed on their leaves. Female *Heliconius* butterflies usually avoid laying eggs on plants already occupied by conspecifics and the presence of these structures mimicking real eggs acts as an oviposition deterrent. Indeed, removal of these egg-like structures was sufficient to increase by 60% the attractiveness of the plant for oviposition by *H. cydno* (Williams and Gilbert, 1981; de Castro et al., 2018). This is demonstrating a fascinating example of indirect defence targeting insect behaviour.

Plant molecular responses to oviposition start progressively to be unveiled. Several studies showed that insect eggs deposition triggers a large transcriptional reprogramming in plants (Little et al., 2007; Firtzlaff et al., 2016; Nallu et al., 2018; Lortzing et al., 2020). In *Arabidopsis*, oviposition by the large white butterfly *Pieris brassicae* (Fig. 1) induces hundreds of stress-related and SA-responsive genes, a transcriptional profile drastically distinct from herbivory-associated responses, which trigger JA-related defences (Little et al., 2007). Accordingly, SA accumulates at high levels upon oviposition or treatment with crude egg extract (EE) at the local site of application as well as to a lesser extent in systemic tissues (Bruessow et al., 2010). Although induction of JA-related defences following oviposition was reported, this seems to be associated with ovipositional wounding, as in *D. pini – Pinus sylvestris* interaction, where eggs are deposited after the female slits the pine needle (Hilker et al., 2002). In addition to SA, *Arabidopsis* induces local accumulation of ROS, cell death and

expression of early responsive genes, which are responses commonly activated after pathogen detection during PTI (Gouhier-Darimont et al., 2013) (Fig. 2). Moreover, several SA biosynthetic and regulatory genes such as *ICS1, NPR1, EDS1* were shown to be essential for egg-induced responses, providing further similarities with PTI responses (Gouhier-Darimont et al., 2013).

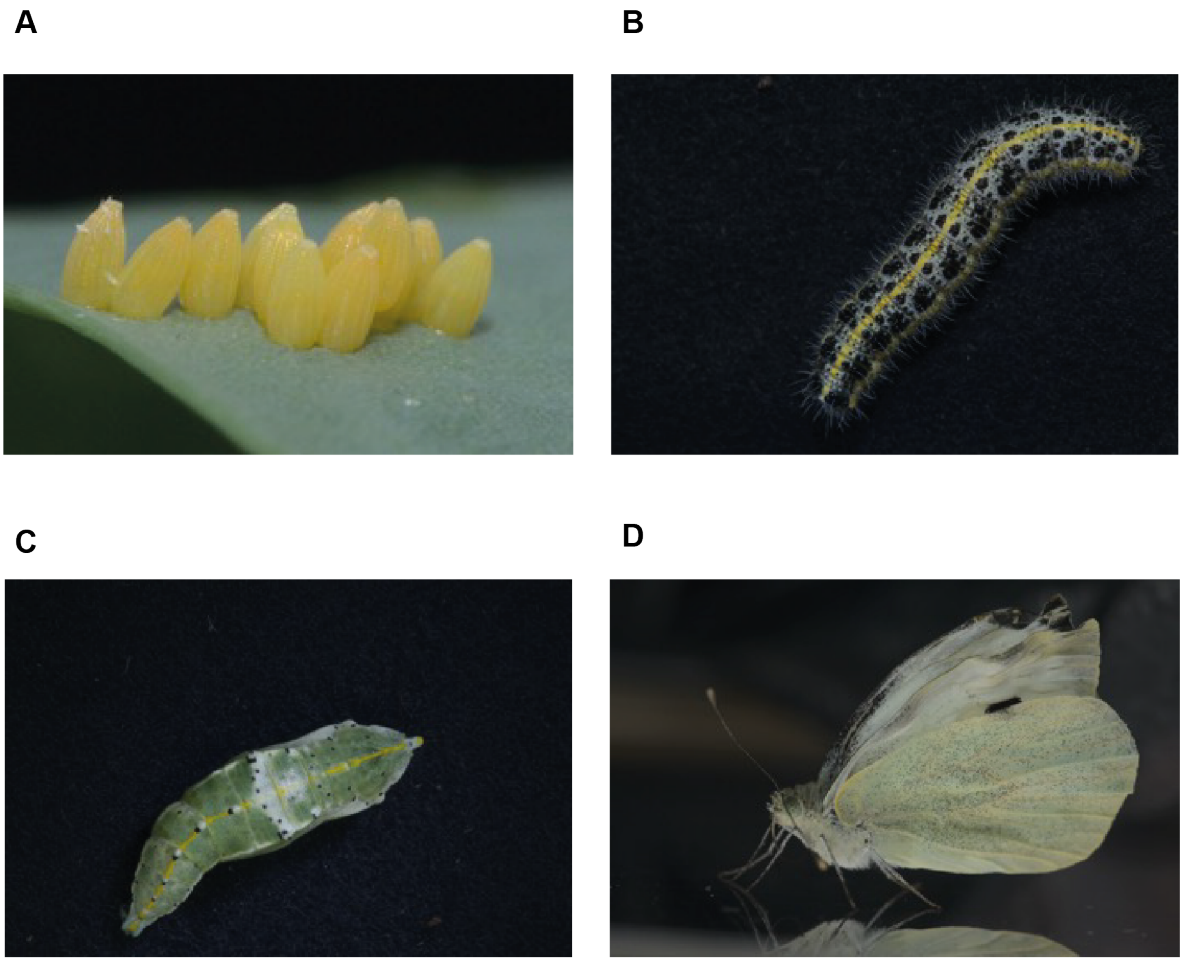


Figure 1. *Pieris brassicae* life stages.
(**A**) Eggs; (**B**) Fourth instar larva; (**C**) Young chrysalis; (**D**) Butterfly
Photographs courtesy of Zigmunds Orlovskis.

To induce any responses, egg-derived elicitor(s) should be delivered and recognized by the plant. The first identified egg-associated molecular patterns (EAMPs) were actually found in egg-associated secretions or in adult insects. Benzyl cyanide is a male-derived anti-aphrodisiac molecule found in female accessory reproductive gland secretions and are released with eggs from *P. brassicae*. This elicitor triggers leaf surface chemical changes and is

responsible for the arrest of the egg parasitoid *Trichogramma brassicae* (Fatouros et al., 2008). Proteins or peptides from oviduct secretions that are released with eggs from the pine sawfly *D. pini* and the elm leaf beetle *X. luteola* also act as elicitors by inducing OIPVs emission (Hilker et al., 2005). Most of insect eggs contain a considerable amount of lipids and proteins in order to support embryogenesis. Seeking for egg-derived elicitor(s) contained inside *P. brassicae* eggs, initial experiments using an *Arabidopsis* transgenic line expressing the β-glucuronidase (*GUS*) gene coupled to the promoter of the SA-marker gene *PATHOGENESIS-RELATED 1* (*PR1*) were conducted (Bruessow et al., 2010). It was shown that crude EE triggers a strong and localized expression of *PR1* (Bruessow et al., 2010). This eliciting activity is even enriched when a fraction containing total egg lipids is applied (Bruessow et al., 2010; Gouhier-Darimont et al., 2013). Solid-phase extraction and nuclear magnetic resonance analyses of this EE-derived lipid fraction allowed to identify phosphatidylcholines (PCs) as being the most abundant lipid species contained in EE (Stahl et al., 2020). It was demonstrated that these PCs can diffuse out of the eggs and induce SA accumulation, cell death, H_2O_2 production and defence gene expression similarly to oviposition and EE application (Stahl et al., 2020). Interestingly, a similar concentration of PCs is contained in EE from *S. littoralis*, which was shown to induce the same responses than *P. brassicae* EE (Bruessow et al., 2010; Stahl et al., 2020). Intriguingly, extracts from adult females of *Sogatella furcifera* also contain PCs, which trigger accumulation of the ovicidal substance benzyl benzoate in rice, although their occurrence in eggs has not been demonstrated (Yang et al., 2014). Overall, these findings support PCs as being active EAMPs that can be released, recognised, and elicit various immune responses.

Generally, plants detect elicitors via specific receptors localized at the plasma membrane. In an attempt to identify such receptor, 41 T-DNA lines for different egg-induced RLKs were screened by measuring *PR1* expression following EE application (Little et al., 2007; Gouhier-Darimont et al., 2013). Only one line carrying a mutation in *LecRK-I.8*, a L-type lectin receptor kinase, was shown to display strongly reduced *PR1* expression in response to EE treatment (Gouhier-Darimont et al., 2013). Further experiments showed that egg-induced ROS production, cell death and SA accumulation were strongly reduced in *lecrk-I.8* mutant, suggesting that LecRK-I.8 is a crucial component of egg perception (Gouhier-Darimont et al., 2019). *LecRK-I.8* is part of a subclade of 11 *LecRK* genes that are closely related to each other (Bellande et al., 2017). Egg-induced responses were not completely abolished in *lecrk-I.8* single mutant and it cannot be excluded that some redundancy by the other subclade members might contribute to the residual response (Gouhier-Darimont et al., 2019). Interestingly, it was

recently found that LecRK-I.1 is specifically involved in the regulation of egg-induced HR-like response and a *lecrk-I.1* mutant displayed reduced responses to EE (Groux et al., 2020). Finally, above-mentioned egg-induced responses were strongly reduced in *lecrk-I.8* mutant when treated with PCs similarly to oviposition and EE treatment, reinforcing the discovery of PCs as active EAMPs (Stahl et al., 2020). However, whether LecRK-I.8 and/or LecRK-I.1 can bind to PCs is currently unknown.

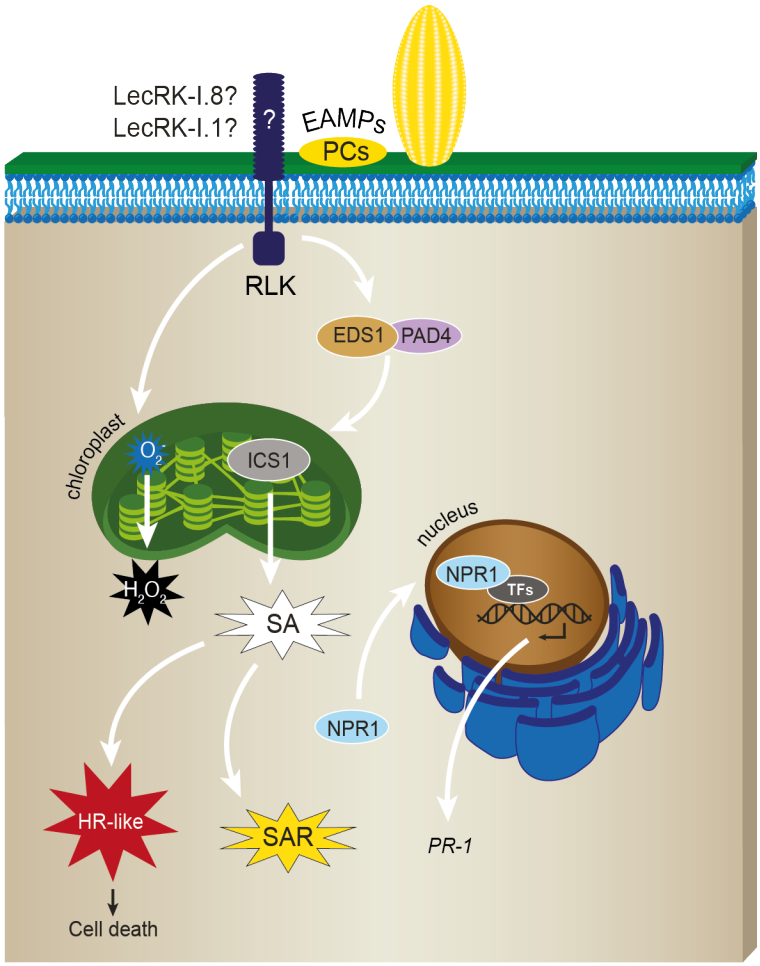


Figure 2. Simplified model of the molecular signalling events following *P. brassicae* oviposition in *Arabidopsis*. Refer to the text for details. Modified from Stahl et al. (2018) with permission.
EAMP, egg-associated molecular pattern; PC, phosphatidylcholine; RLK, receptor-like kinase; SA, salicylic acid; SAR, systemic acquired resistance; HR, hypersensitive response; TF, transcription factor.

Beside directly impacting egg survival, egg-induced responses can also affect defences against future hatching larvae, both negatively and positively. Upon *P. brassicae* oviposition, the black mustard *Brassica nigra* emits volatiles that attract the larval parasitoid *Cotesia glomerata*, a process considered as an egg-induced indirect defence against herbivory (Fatouros et al., 2012). Several studies demonstrated that insect eggs can be perceived as a signal for imminent herbivory and that they can prime anti-herbivore defences. Larvae of the generalist *Spodoptera exigua* performed worse on oviposited *Nicotiana attenuata* and *S. dulcamara* in comparison to egg-free plants, resulting from priming of feeding-induced defence traits by prior oviposition (Bandoly et al., 2015; Geuss et al., 2018). In *Arabidopsis*, treatment with *S. littoralis* or *P. brassicae* EE was shown to inhibit feeding-induced JA-related defences. It is known that SA and JA pathways act antagonistically (Pieterse et al., 2009). Following EE treatment, performance of *S. littoralis* was enhanced and this was dependent on SA accumulation, suggesting that eggs may hijack the SA pathway for the benefit of their progeny. However, *P. brassicae* larvae did not benefit from reduced defences, probably due to the tolerance of this insect towards *Arabidopsis* defences (Wittstock et al., 2004). On the contrary, other studies found that *P. brassicae* fed less on previously oviposited *Arabidopsis* and exhibited higher mortality (Geiselhardt et al., 2013; Pashalidou et al., 2013; Valsamakis et al., 2020). Surprisingly, this effect was SA-dependent and was associated with flavonoid metabolism (Lortzing et al., 2019). Discrepancies among these studies might be due to the type of plant treatment (EE application versus natural oviposition), although it was recently shown that plant responses to both are similar (Stahl et al., 2020), and/or the type of larval feeding (single larva versus gregariously feeding behaviour). In addition to SA-JA crosstalk, egg-induced SA accumulation might also impact pre-existing or further plant-pathogen interactions.

Systemic acquired resistance

Systemic acquired resistance (SAR) is an inducible leaf-to-leaf defence response triggered after a primary localized pathogen infection. This results in enhanced defences in the whole foliage, providing resistance against a broad range of pathogens (Fu and Dong, 2013; Shah and Zeier, 2013; Vlot et al., 2020). Another form of systemic immunity is triggered in roots by beneficial soil microorganisms and induce resistance in aerial parts of the plant, a process termed induced systemic resistance (ISR) (Pieterse et al., 2014). The first observation of SAR was reported when uninfected systemic leaves of tobacco exhibited increased immunity following initial infection with tobacco mosaic virus (Ross, 1961). SAR requires the SA pathway, the generation and translocation of a mobile signal and is accompanied with priming of defence gene expression in systemic tissues (Shah and Zeier, 2013; Vlot et al., 2020). SA was thought to be the SAR mobile signal because it was accumulating at high levels in local infected leaves as well as in petiole exudate of such leaves (Malamy et al., 1990; Metraux et al., 1990). However, grafting experiments showed that SA-deficient tobacco rootstocks were still able to transmit a signal and activate SAR in wild-type tobacco scions, suggesting that SA is not the transmitted signal but is rather essential for SAR establishment in systemic tissues (Gaffney et al., 1993; Vernooij et al., 1994). Further studies identified several putative SAR signals, such as methyl salicylate (MeSA), azelaic acid (AzA), glycerol-3-phosphate (G3P), dehydroabietinal (DA) as well as the non-protein amino acid pipecolic acid (Pip) and its derivative *N*-hydroxy-pipecolic acid (NHP) (Fu and Dong, 2013; Shah and Zeier, 2013; Hartmann and Zeier, 2018; Vlot et al., 2020).

Recent studies have demonstrated that the NHP pathway is essential for SAR establishment. Upon pathogen infection, Pip accumulates at high levels in local and systemic leaves, inducing SA accumulation and defence gene expression (Návarová et al., 2012). The complete biosynthesis of Pip and its derivative NHP has been recently elucidated (Hartmann et al., 2017). AGD2-LIKE DEFENSE RESPONSE PROTEIN 1 (ALD1) encodes a aminotransferase and catalyses the first step of Pip biosynthesis by transferring the α-amino group of L-Lysine (Lys) to acceptor molecules to generate 2,3-dehydropipecolic acid (DP), which is then reduced by SAR-DEFICIENT 4 (SARD4) and possibly other reductases to generate Pip in the chloroplast (Ding et al., 2016; Hartmann et al., 2017) (Fig. 3B). Although ALD1 can use other amino acids than Lys to catalyse transamination *in vitro*, products of such reactions are not detectable *in planta*, suggesting that the biosynthesis of 2,3-DP from Lys is the major function of ALD1 (Hartmann et al., 2017). Accordingly, *ald1* mutant is lacking 2,3-

DP and Pip and is fully SAR-deficient (Návarová et al., 2012; Hartmann et al., 2017). FLAVIN-DEPENDENT MONOOXYGENASE 1 (FMO1) further metabolizes Pip in the cytosol to generate NHP, which acts as the metabolic regulator of SAR (Chen et al., 2018; Hartmann et al., 2018). Consequently, *fmo1* mutant is SAR-deficient and exogenous application of NHP but not Pip is able to restore SAR (Mishina and Zeier, 2006; Chen et al., 2018; Hartmann et al., 2018). Furthermore, it was demonstrated that deuterated NHP can move systemically in exogenously supplied *Arabidopsis* (Mohnike et al., 2021). In addition to NHP, an hexose-conjugated form of NHP was also detected in systemic tissues of SAR-activated plants (Chen et al., 2018). Activity regulation of defence metabolites can be achieved by chemical modifications such as glycosylation, carried out by UDP-glycosyltransferases (UGTs). During pathogen attack, SA accumulates as well as its inactive glycosylated forms, SA glucose ester (SGE) and SA β-glucoside (SAG) (Vlot et al., 2009). It was shown that these conjugates are produced by the action of several UGTs, including UGT76B1, which was also described to glycosylate isoleucic acid (von Saint Paul et al., 2011; Noutoshi et al., 2012). Several independent studies showed that UGT76B1 can glycosylate NHP to generate an NHP-*O*-glucoside (NHPG), which is biologically inactive. Indeed, *ugt76b1* mutant is unable to generate NHPG and displays constitutively active defence responses and a reduced growth phenotype, which is attributed to the overaccumulation of NHP (Bauer et al., 2021; Cai et al., 2021; Holmes et al., 2021; Mohnike et al., 2021). Together, these studies indicate that NHP is the active SAR signal inducing SA accumulation and SAR upon pathogen infection and uncover its regulation mechanism through glycosylation by UGT76B1.

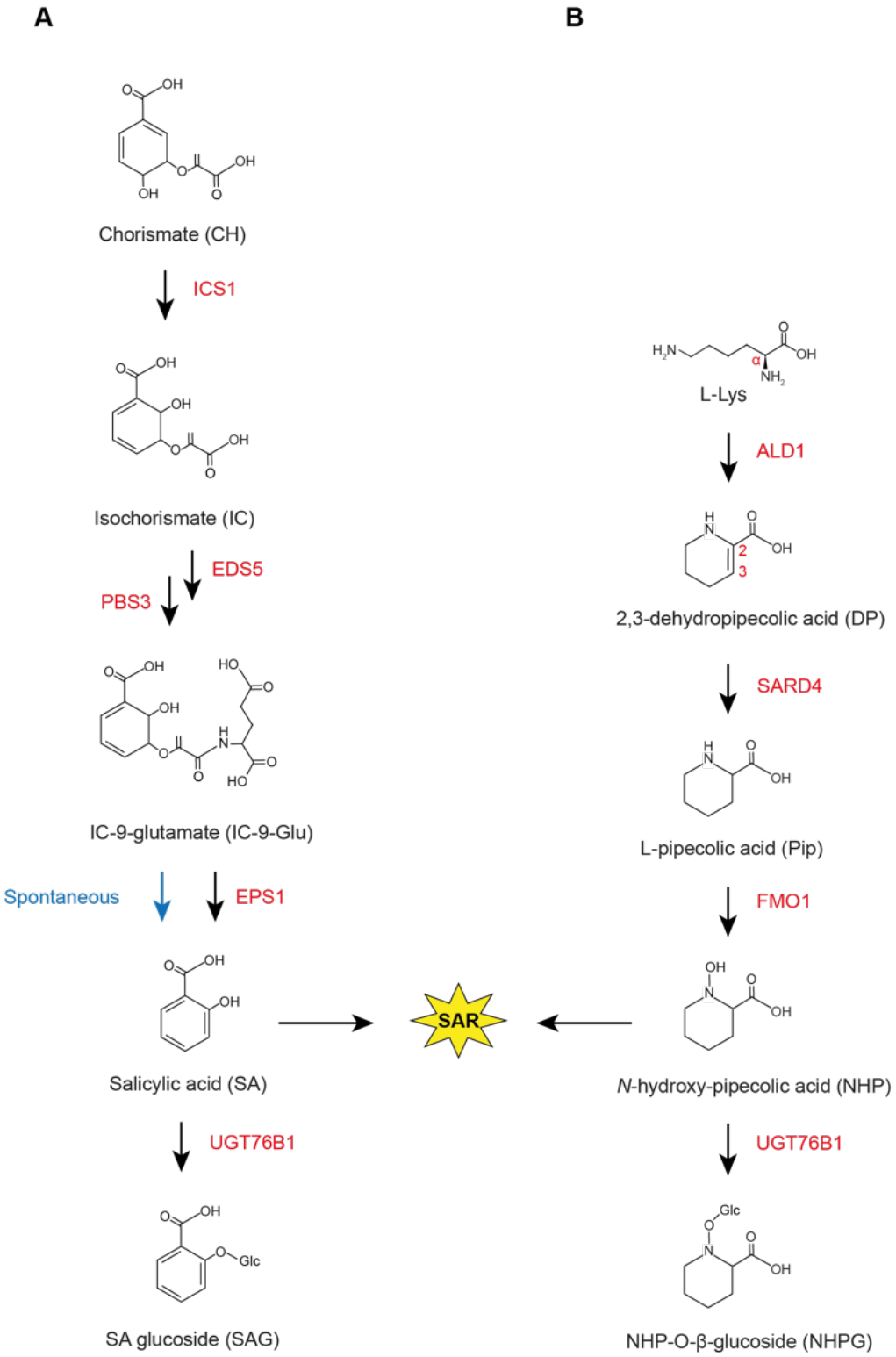


Figure 3. Biosynthesis of SA and NHP in *Arabidopsis*.

(**A**) SA biosynthesis pathway. **(B**) NHP biosynthesis pathway. Refer to the text for details. SAR, systemic acquired resistance.

SA and NHP pathways are inter-connected during SAR (Fig. 3) and biosynthetic genes of both pathways are commonly regulated by several transcription factors (Vlot et al., 2020; Zeier, 2021). The interacting proteins EDS1 and PAD4 have been shown to mediate basal plant resistance by promoting SA biosynthesis (Jirage et al., 1999; Feys et al., 2001). Pip and NHP accumulate in an SA-independent manner at the site of infection, promoted by EDS1/PAD4 signalling (Mishina and Zeier, 2006; Bernsdorff et al., 2016; Hartmann et al., 2018). Consequently, a minor SAR response was still observable in the SA-deficient *ics1* mutant, in an FMO1-dependent manner, suggesting that the NHP pathway can promote SAR in a SA-dependent and independent way (Bernsdorff et al., 2016). Two transcription factors induced during SAR, SAR-DEFICIENT 1 (SARD1) and CALMODULIN-BINDING PROTEIN 60g (CBP60g), activate biosynthesis of SA and NHP through direct binding to the promoters of *ICS1, ALD1* and *FMO1* (Wang et al., 2011; Sun et al., 2015). Consequently, the *sard1 cbp60g* double mutant has reduced levels of SA and Pip and is SAR-deficient (Zhang et al., 2010; Sun et al., 2018). *SARD1* and *CBP60g* are positively regulated by several proteins, including EDS1/PAD4 and TGA1 and TGA4 (Gruner et al., 2013; Sun et al., 2018). When exogenously applied, Pip induces, in a FMO1-dependent manner, the expression of genes regulating SAR such as *ALD1, SARD4, FMO1, ICS1, EDS5, EDS1, PAD4, SARD1* and *CBP60g* (Hartmann et al., 2018), NHP application induces *ALD1, SARD4, FMO1* and *ICS1* expression (Chen et al., 2018). SA accumulation has also been shown to induce *SARD1, EDS1, PAD4* and *SARD4* expression, highlighting the ability of these metabolites to positively regulate their own biosynthesis as well as various SAR-promoting genes (Hartmann and Zeier, 2019). Another regulation of the NHP pathway occurs through the action of the Pip-inducible transcription factor WRKY33. It was shown recently that in addition to its role in promoting camalexin biosynthesis (Mao et al., 2011), WRKY33 is positively regulating NHP biosynthesis and therefore SAR establishment by binding to the promoter of *ALD1* (Wang et al., 2018). WRKY33 is directly phosphorylated by MPK3 and MPK6 as well as CPK5 and CPK6, which were also shown to activate SARD1, illustrating a common positive regulation of camalexin biosynthesis and NHP pathway for SAR establishment (Mao et al., 2011; Wang et al., 2018; Guerra et al., 2020; Zhou et al., 2020) (Fig. 4).

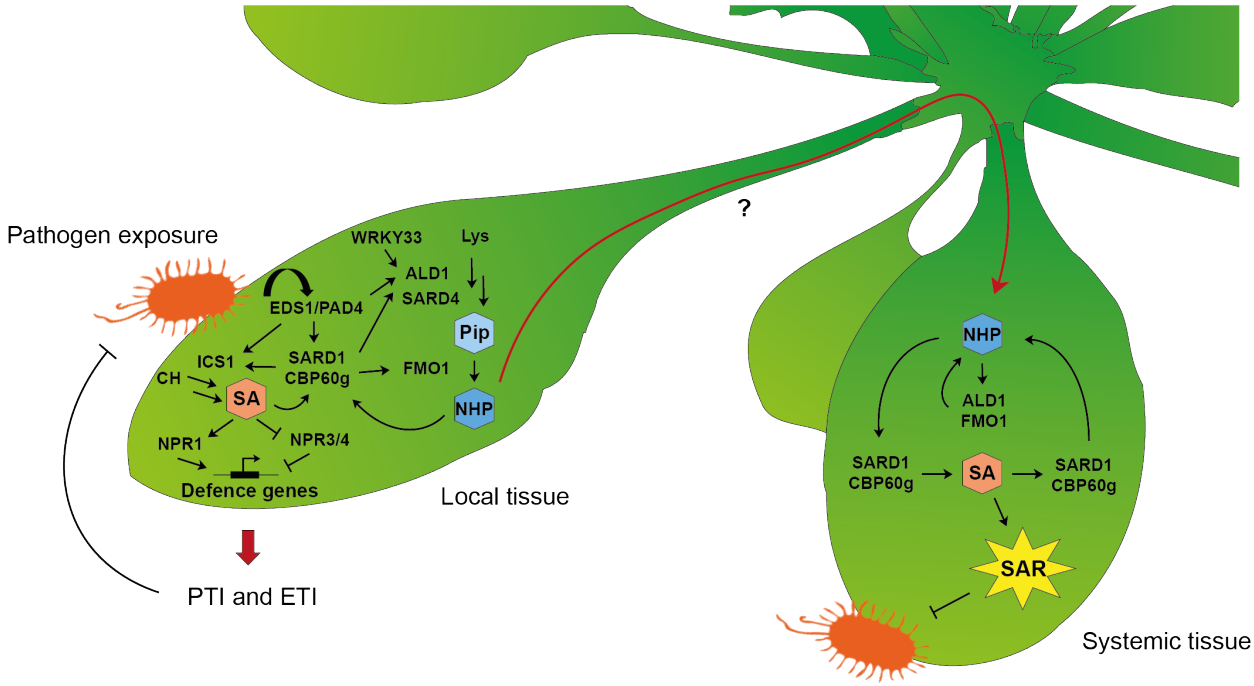


Figure 4. Model depicting SAR signalling.
Refer to the text for details. Arrows indicate positive regulation. Blunt end lines indicate inhibition. Multiple arrows indicate several steps. Abbreviations: CH: chorismate; SA: salicylic acid; PTI: pattern-triggered immunity; ETI: effector-triggered immunity; Lys: lysine; Pip: pipecolic acid; NHP: *N*-hydroxy-pipecolic acid; SAR: systemic acquired resistance

Amino acid-related metabolism has important functions in plant immunity (Zeier, 2013). Tryptophan (Trp)-derived metabolism constitutes an important reservoir of indolic compounds with defence-relevant activity against a broad range of pathogens and herbivores in *Arabidopsis* and other crucifers (Bednarek et al., 2011; Bednarek, 2012; Kettles et al., 2013; Rajniak et al., 2015). Trp is first converted by the two cytochrome P450 enzymes CYP79B2 and CYP79B3 to generate indole-3-acetaldoxime (IAOx) (Zhao et al., 2002). From IAOx, several branches diverge to generate indole glucosinolates, camalexin, indole-3-carboxylic acid (ICA), indole-3-acetic acid and other small indolic metabolites (Glawischnig, 2007; Bender and Celenza, 2009; Bednarek, 2012) (Fig. 5). Moreover, during bacterial-induced SAR, ICA, indole-3-carbaldehyde and indole-3-ylmethylamine accumulated in systemic leaves, illustrating a connection between Trp-derived indolic metabolism and SAR in *Arabidopsis* (Stahl et al., 2016).

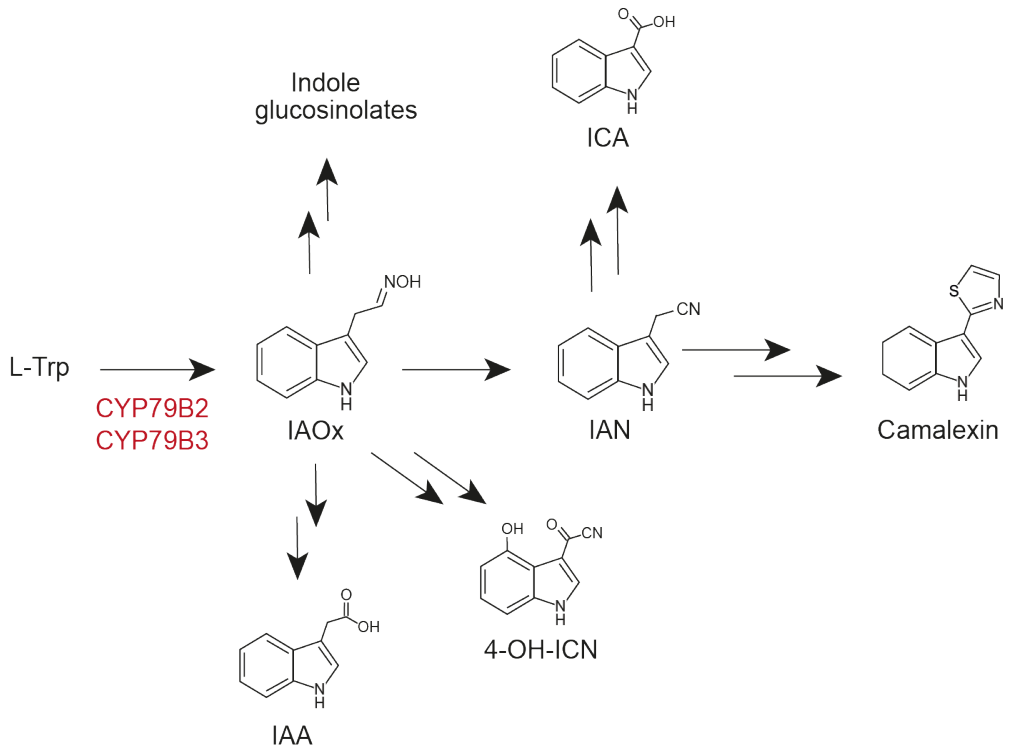


Figure 5. Simplified scheme illustrating tryptophan-derived indolic metabolism.
Multiple arrows indicate multiple biosynthesis steps. L-Trp, tryptophan; IAOx, indole-3-acetaldoxime; IAA, indole-3-acetic acid; 4-OH-ICN, 4-hydroxy-indole-3-carbonyl nitrile; IAN, indole-3-acetonitrile; ICA, indole-3-carboxylic acid.

Recently, it was found that *P. brassicae* oviposition induces a SAR against the bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*) in *Arabidopsis* (Hilfiker et al., 2014). This egg-induced SAR was dependent on a functional NHP pathway, suggesting a conserved mechanism with pathogen-induced SAR (Hilfiker et al., 2014). By reducing pathogen load on leaves, egg-induced SAR might be beneficial for hatching larvae and it was shown that indeed, *P. brassicae* larvae performed better on *Pst*-infected plants previously treated with EE compared to *Pst*-infected plants (Hilfiker et al., 2014). It was demonstrated in *Nicotiana benthamiana* that SAR can be transmissible to neighbouring plants via a root-mediated pathway (Cheol Song et al., 2016). Egg-induced interplant SAR against *Pst* was recently discovered in *Arabidopsis* and was shown to occur via a mobile root-derived signal in an *ALD1*- and *FMO1*-dependent manner (Orlovskis and Reymond, 2020). Ecological functions of such egg-induced responses are still unclear, but it can be hypothesized that reducing pathogen load in neighbouring plants might be beneficial for the development of larvae. Indeed, larvae are moving and changing hosts when levels of induced defences are too high and such

protection of neighbouring plants might increase their chances of survival. However, whether such insect egg-induced interplant SAR is occurring in natural environment and have fitness benefits for both insects and plants remains to be studied.

Arabidopsis – Botrytis cinerea pathosystem

The ascomycete *Botrytis cinerea* is a necrotrophic plant pathogen causing the grey mould disease, which is characterized by the rotting of all aerial parts of plants to produce abundant grey conidiophores and conidia (Fig. 6). *B. cinerea* is spread worldwide and can infect more than 1000 species, including many important crops and is therefore considered as the second most important plant fungal pathogen (Williamson et al., 2007; Dean et al., 2012). For a long time, it was considered that *B. cinerea* infection strategies mainly rely on deployment of cell wall-degrading enzymes, ROS and phytotoxic compounds (van Kan, 2006), but recent studies showed that this fungus uses subtler means to infect its hosts. Indeed, *B. cinerea* can exploit biological processes in hosts for its own success. It has been shown that *B. cinerea* silences *Arabidopsis* genes involved in immunity through the translocation of small RNAs (sRNA) that hijack the plant RNA interference (RNAi) pathway (Weiberg et al., 2013). This process occurs in the early phase of the infection and requires plant hosts to be alive, suggesting an early and short biotrophic lifestyle of *B. cinerea*, placing it in the category of hemibiotrophic rather than necrotrophic fungi (Veloso and van Kan, 2018). This cross-kingdom RNAi mechanism can even occur in a bi-directional manner, since transgenic *Arabidopsis* and tomato plants expressing sRNAs targeting the RNAi pathway of *B. cinerea* were shown to exhibit increased resistance (Wang et al., 2016). Early works showed that infection of *Arabidopsis* with *B. cinerea* leads to the induction of many defence genes that are mainly regulated by JA and ET signalling pathways. Mutants impaired in JA and ET signalling are highly susceptible to *B. cinerea* infection (Thomma et al., 1999). There are however reports pointing to a contribution of SA signalling pathway. Exogenous SA application decreases *B. cinerea* lesion size and the ET insensitive mutant *ein2-1* is more resistant than the *ein2-1 npr1* double mutant (Ferrari et al., 2003). Furthermore, recent phenotypic and transcriptomic analyses of *Arabidopsis* plants infected with various *B. cinerea* isolates support a more intricated role of JA and SA pathways in resistance (Zhang et al., 2017). In addition to this, plant defences as well as pathogen virulence have been shown to fluctuate during the day (Sharma and Bhatt, 2015). *Arabidopsis* susceptibility to *B. cinerea* was decreased when infection occurred at dawn and this was associated with a faster expression of JA/ET-responsive genes at that time of the day (Ingle et

al., 2015). On the other side, *B. cinerea* circadian clock regulates its pathogenicity, with a greater virulence when infection occurs at dusk (Hevia et al., 2015).

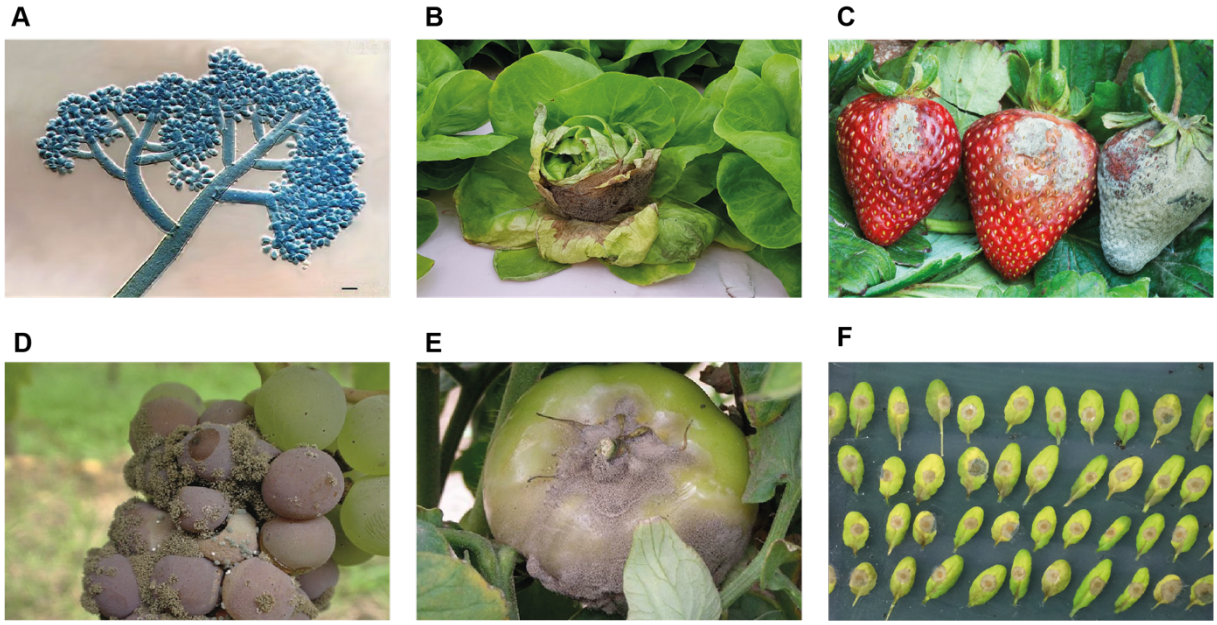


Figure 6. *Botrytis cinerea* and grey mould disease.
(**A**) *B. cinerea* hyphae with conidiophores and conidia. (**B** to **F**) Typical grey mould disease on leaves and fruits of lettuce, strawberries, grape, tomato and *Arabidopsis*, respectively. Pictures were taken from Google Images.

THESIS OUTLINE

As mentioned, *P. brassicae* oviposition and treatment with EE induce a SAR against several strains of the pathogenic bacteria *P. syringae*. This response relies on SA and NHP pathways, which are known SAR regulators, and on priming of defence gene expression. However, no link with secondary metabolism was established. In this study, we investigate whether EE-induced SAR is extended to other pathogens, and we found that it also reduces *B. cinerea* growth in systemic leaves. We then focused on the involvement of indolic metabolism in this response, which is a pathway induced following infection by various pathogens. Finally, we addressed the biological relevance of this response with various bioassays.

In Chapter 1 of this thesis, we investigate whether EE-induced SAR against *B. cinerea* relies on similar signalling mechanisms than EE-induced SAR against *P. syringae*. By performing bioassays with several signalling mutants, we demonstrate that SA and NHP pathways are also required to establish SAR against *B. cinerea*. Chapter 2 reports the involvement of tryptophan-derived indolic metabolism in this response, with a substantial role of camalexin, an important defence metabolite in *Arabidopsis*. In Chapter 3, we show that glucose conjugated derivatives of indole-3-carbaldehyde and indole-3-carboxylic acid accumulate in systemic leaves of EE-treated plants. This response is lost in the Pip-deficient mutant *ald1* and in the indole glucosinolates triple mutant *myb34 myb51 myb122* but conserved in other indolic mutants. These metabolites have no function in EE-induced SAR establishment against *B. cinerea*, but we discuss their potential involvement in SAR against other pathogens. Finally, Chapter 4 focuses on the biological relevance of EE-induced SAR. Performing bioassays measuring *P. brassicae* larval performance on *B. cinerea*-infected plants, we show that development of the insect is impacted. In addition, we also demonstrate that treatment with EE locally reduces *B. cinerea* growth on other *Brassicaceae* plant species.

REFERENCES

Arimura G (2021) Making Sense of the Way Plants Sense Herbivores. Trends in Plant Science **26**: 288–298

Balbyshev NF, Lorenzen JH (1997) Hypersensitivity and Egg Drop: A Novel Mechanism of Host Plant Resistance to Colorado Potato Beetle (Coleoptera: Chrysomelidae). Journal of Economic Entomology **90**: 652–657

Bandoly M, Hilker M, Steppuhn A (2015) Oviposition by *Spodoptera exigua* on *Nicotiana attenuata* primes induced plant defence against larval herbivory. Plant J **83**: 661–672

Bari R, Jones JDG (2009) Role of plant hormones in plant defence responses. Plant Mol Biol **69**: 473–488

Bauer S, Mekonnen DW, Hartmann M, Yildiz I, Janowski R, Lange B, Geist B, Zeier J, Schaffner AR (2021) UGT76B1, a promiscuous hub of small molecule-based immune signaling, glucosylates N-hydroxypipecolic acid, and balances plant immunity. The Plant Cell **0**: 21

Bednarek P (2012) Chemical warfare or modulators of defence responses – the function of secondary metabolites in plant immunity. Current Opinion in Plant Biology **15**: 407–414

Bednarek P, Pislewska-Bednarek M, Svatos A, Schneider B, Doubsky J, Mansurova M, Humphry M, Consonni C, Panstruga R, Sanchez-Vallet A, et al (2009) A Glucosinolate Metabolism Pathway in Living Plant Cells Mediates Broad-Spectrum Antifungal Defense. Science **323**: 101–106

Bednarek P, Piślewska-Bednarek M, Ver Loren van Themaat E, Maddula RK, Svatoš A, Schulze-Lefert P (2011) Conservation and clade-specific diversification of pathogen-inducible tryptophan and indole glucosinolate metabolism in Arabidopsis thaliana relatives. New Phytologist **192**: 713–726

Bellande K, Bono J-J, Savelli B, Jamet E, Canut H (2017) Plant Lectins and Lectin Receptor-Like Kinases: How Do They Sense the Outside? IJMS **18**: 1164

Bender J, Celenza JL (2009) Indolic glucosinolates at the crossroads of tryptophan metabolism. Phytochem Rev 13

Bernsdorff F, Döring A-C, Gruner K, Schuck S, Bräutigam A, Zeier J (2016) Pipecolic Acid Orchestrates Plant Systemic Acquired Resistance and Defense Priming via Salicylic Acid-Dependent and -Independent Pathways. Plant Cell **28**: 102–129

Bertea CM, Casacci LP, Bonelli S, Zampollo A, Barbero F (2020) Chemical, Physiological and Molecular Responses of Host Plants to Lepidopteran Egg-Laying. Front Plant Sci **10**: 1768

Bonnet C, Lassueur S, Ponzio C, Gols R, Dicke M, Reymond P (2017) Combined biotic stresses trigger similar transcriptomic responses but contrasting resistance against a chewing herbivore in Brassica nigra. BMC Plant Biol **17**: 127

Bruessow F, Gouhier-Darimont C, Buchala A, Metraux J-P, Reymond P (2010) Insect eggs suppress plant defence against chewing herbivores: Insect eggs and plant defence. The Plant Journal **62**: 876–885

Büchel K, Malskies S, Mayer M, Fenning TM, Gershenzon J, Hilker M, Meiners T (2011) How plants give early herbivore alert: Volatile terpenoids attract parasitoids to egg-infested elms. Basic and Applied Ecology **12**: 403–412

Bürger M, Chory J (2019) Stressed Out About Hormones: How Plants Orchestrate Immunity. Cell Host & Microbe **26**: 163–172

Cai J, Jozwiak A, Holoidovsky L, Meijler MM, Meir S, Rogachev I, Aharoni A (2021) Glycosylation of N-hydroxy-pipecolic acid equilibrates between systemic acquired resistance response and plant growth. Molecular Plant **14**: 440–455

de Castro ÉCP, Zagrobelny M, Cardoso MZ, Bak S (2018) The arms race between heliconiine butterflies and *Passiflora* plants - new insights on an ancient subject: New insights on an ancient subject. Biol Rev **93**: 555–573

Chen Y-C, Holmes EC, Rajniak J, Kim J-G, Tang S, Fischer CR, Mudgett MB, Sattely ES (2018) *N* -hydroxy-pipecolic acid is a mobile metabolite that induces systemic disease resistance in *Arabidopsis*. Proc Natl Acad Sci USA **115**: E4920–E4929

Cheol Song G, Sim H-J, Kim S-G, Ryu C-M (2016) Root-mediated signal transmission of systemic acquired resistance against above-ground and below-ground pathogens. Ann Bot **118**: 821–831

Chini A, Fonseca S, Fernández G, Adie B, Chico JM, Lorenzo O, García-Casado G, López-Vidriero I, Lozano FM, Ponce MR, et al (2007) The JAZ family of repressors is the missing link in jasmonate signalling. Nature **448**: 666–671

Couto D, Zipfel C (2016) Regulation of pattern recognition receptor signalling in plants. Nat Rev Immunol **16**: 537–552

Dean R, Van Kan JAL, Pretorius ZA, Hammond-Kosack KE, Di Pietro A, Spanu PD, Rudd JJ, Dickman M, Kahmann R, Ellis J, et al (2012) The Top 10 fungal pathogens in molecular plant pathology: Top 10 fungal pathogens. Molecular Plant Pathology **13**: 414–430

Desurmont GA, Donoghue MJ, Clement WL, Agrawal AA (2011) Evolutionary history predicts plant defense against an invasive pest. Proceedings of the National Academy of Sciences **108**: 7070–7074

Deutsch CA, Tewksbury JJ, Tigchelaar M, Battisti DS, Merrill SC, Huey RB, Naylor RL (2018) Increase in crop losses to insect pests in a warming climate. Science **361**: 916–919

Ding P, Rekhter D, Ding Y, Feussner K, Busta L, Haroth S, Xu S, Li X, Jetter R, Feussner I, et al (2016) Characterization of a Pipecolic Acid Biosynthesis Pathway Required for Systemic Acquired Resistance. Plant Cell **28**: 2603–2615

Ding Y, Sun T, Ao K, Peng Y, Zhang Y, Li X, Zhang Y (2018) Opposite Roles of Salicylic Acid Receptors NPR1 and NPR3/NPR4 in Transcriptional Regulation of Plant Immunity. Cell **173**: 1454-1467.e15

Doss RP, Oliver JE, Proebsting WM, Potter SW, Kuy S, Clement SL, Williamson RT, Carney JR, DeVilbiss ED (2000) Bruchins: Insect-derived plant regulators that stimulate neoplasm formation. Proceedings of the National Academy of Sciences **97**: 6218–6223

Douglas AE (2018) Strategies for Enhanced Crop Resistance to Insect Pests. Annu Rev Plant Biol **69**: 637–660

Erb M, Reymond P (2019) Molecular Interactions Between Plants and Insect Herbivores. Annu Rev Plant Biol **70**: 527–557

Fatouros NE, Broekgaarden C, Bukovinszkine'Kiss G, van Loon JJA, Mumm R, Huigens ME, Dicke M, Hilker M (2008) Male-derived butterfly anti-aphrodisiac mediates induced indirect plant defense. Proceedings of the National Academy of Sciences **105**: 10033–10038

Fatouros NE, Bukovinszkine'Kiss G, Kalkers LA, Gamborena RS, Dicke M, Hilker M (2005) Oviposition-induced plant cues: do they arrest *Trichogramma* wasps during host location? Entomologia Experimentalis et Applicata **115**: 207–215

Fatouros NE, Lucas-Barbosa D, Weldegergis BT, Pashalidou FG, van Loon JJA, Dicke M, Harvey JA, Gols R, Huigens ME (2012) Plant Volatiles Induced by Herbivore Egg Deposition Affect Insects of Different Trophic Levels. PLoS ONE **7**: e43607

Ferrari S, Plotnikova JM, De Lorenzo G, Ausubel FM (2003) *Arabidopsis* local resistance to *Botrytis cinerea* involves salicylic acid and camalexin and requires *EDS4* and *PAD2* , but not *SID2* , *EDS5* or *PAD4*: *Local* Arabidopsis *response to* Botrytis. The Plant Journal **35**: 193–205

Feys BJ, Moisan LJ, Newman M-A, Parker JE (2001) Direct interaction between the Arabidopsis disease resistance signaling proteins, EDS1 and PAD4. The EMBO Journal **20**: 5400–5411

Firtzlaff V, Oberländer J, Geiselhardt S, Hilker M, Kunze R (2016) Pre-exposure of Arabidopsis to the abiotic or biotic environmental stimuli "chilling" or "insect eggs" exhibits different transcriptomic responses to herbivory. Sci Rep **6**: 28544

Fonseca S, Chini A, Hamberg M, Adie B, Porzel A, Kramell R, Miersch O, Wasternack C, Solano R (2009) (+)-7-iso-Jasmonoyl-L-isoleucine is the endogenous bioactive jasmonate. Nat Chem Biol **5**: 344–350

Fu ZQ, Dong X (2013) Systemic Acquired Resistance: Turning Local Infection into Global Defense. Annu Rev Plant Biol **64**: 839–863

Fu ZQ, Yan S, Saleh A, Wang W, Ruble J, Oka N, Mohan R, Spoel SH, Tada Y, Zheng N, et al (2012) NPR3 and NPR4 are receptors for the immune signal salicylic acid in plants. Nature **486**: 228–232

Gaffney T, Friedrich L, Vernooij B, Negrotto D, Nye G, Uknes S, Ward E, Kessmann H, Ryals J (1993) Requirement of Salicylic Acid for the Induction of Systemic Acquired Resistance. Science **261**: 754–756

Gagic V, Holding M, Venables WN, Hulthen AD, Schellhorn NA (2021) Better outcomes for pest pressure, insecticide use, and yield in less intensive agricultural landscapes. Proc Natl Acad Sci USA **118**: e2018100118

Garcion C, Lohmann A, Lamodière E, Catinot J, Buchala A, Doermann P, Métraux J-P (2008) Characterization and Biological Function of the *ISOCHORISMATE SYNTHASE2* Gene of Arabidopsis. Plant Physiol **147**: 1279–1287

Geiselhardt S, Yoneya K, Blenn B, Drechsler N, Gershenzon J, Kunze R, Hilker M (2013) Egg Laying of Cabbage White Butterfly (Pieris brassicae) on Arabidopsis thaliana Affects Subsequent Performance of the Larvae. PLoS ONE **8**: e59661

Geuss D, Lortzing T, Schwachtje J, Kopka J, Steppuhn A (2018) Oviposition by Spodoptera exigua on Solanum dulcamara Alters the Plant's Response to Herbivory and Impairs Larval Performance. IJMS **19**: 4008

Geuss D, Stelzer S, Lortzing T, Steppuhn A (2017) *Solanum dulcamara* 's response to eggs of an insect herbivore comprises ovicidal hydrogen peroxide production: *Solanum dulcamara* 's response to eggs of an insect herbivore. Plant, Cell & Environment **40**: 2663–2677

Glawischnig E (2007) Camalexin. Phytochemistry **68**: 401–406

Glazebrook J (2005) Contrasting Mechanisms of Defense Against Biotrophic and Necrotrophic Pathogens. Annu Rev Phytopathol **43**: 205–227

Gouhier-Darimont C, Schmiesing A, Bonnet C, Lassueur S, Reymond P (2013) Signalling of Arabidopsis thaliana response to Pieris brassicae eggs shares similarities with PAMP-triggered immunity. Journal of Experimental Botany **64**: 665–674

Gouhier-Darimont C, Stahl E, Glauser G, Reymond P (2019) The Arabidopsis Lectin Receptor Kinase LecRK-I.8 Is Involved in Insect Egg Perception. Front Plant Sci **10**: 623

Govrin EM, Levine A (2000) The hypersensitive response facilitates plant infection by the necrotrophic pathogen Botrytis cinerea. Current Biology **10**: 751–757

Griese E, Caarls L, Bassetti N, Mohammadin S, Verbaarschot P, Bukovinszkine'Kiss G, Poelman EH, Gols R, Schranz ME, Fatouros NE (2021) Insect egg-killing: a new front on the evolutionary arms-race between brassicaceous plants and pierid butterflies. New Phytol **230**: 341–353

Groux R, Stahl E, Gouhier-Darimont C, Kerdaffrec E, Jimenez-Sandoval P, Santiago J, Reymond P (2020) Arabidopsis natural variation in insect egg-induced cell death reveals a role for LECTIN RECEPTOR KINASE-I.1. Plant Physiology kiaa022

Gruner K, Griebel T, Návarová H, Attaran E, Zeier J (2013) Reprogramming of plants during systemic acquired resistance. Front Plant Sci. doi: 10.3389/fpls.2013.00252

Guerra T, Schilling S, Hake K, Gorzolka K, Sylvester F, Conrads B, Westermann B, Romeis T (2020) Calcium-dependent protein kinase 5 links calcium signaling with *N* -hydroxy- L -pipecolic acid- and *SARD 1* -dependent immune memory in systemic acquired resistance. New Phytol **225**: 310–325

Haribal M, Renwick JAA (1996) Oviposition stimulants for the monarch butterfly: Flavonol glycosides from Asclepias curassavica. Phytochemistry **41**: 139–144

Hartmann M, Kim D, Bernsdorff F, Ajami-Rashidi Z, Scholten N, Schreiber S, Zeier T, Schuck S, Reichel-Deland V, Zeier J (2017) Biochemical Principles and Functional Aspects of Pipecolic Acid Biosynthesis in Plant Immunity. Plant Physiol **174**: 124–153

Hartmann M, Zeier J (2018) L -lysine metabolism to *N* -hydroxypipecolic acid: an integral immune-activating pathway in plants. Plant J **96**: 5–21

Hartmann M, Zeier J (2019) N-hydroxypipecolic acid and salicylic acid: a metabolic duo for systemic acquired resistance. Current Opinion in Plant Biology **50**: 44–57

Hartmann M, Zeier T, Bernsdorff F, Reichel-Deland V, Kim D, Hohmann M, Scholten N, Schuck S, Bräutigam A, Hölzel T, et al (2018) Flavin Monooxygenase-Generated N-Hydroxypipecolic Acid Is a Critical Element of Plant Systemic Immunity. Cell **173**: 456-469.e16

Hevia MA, Canessa P, Müller-Esparza H, Larrondo LF (2015) A circadian oscillator in the fungus *Botrytis cinerea* regulates virulence when infecting *Arabidopsis thaliana*. Proc Natl Acad Sci USA **112**: 8744–8749

Hilfiker O, Groux R, Bruessow F, Kiefer K, Zeier J, Reymond P (2014) Insect eggs induce a systemic acquired resistance in Arabidopsis. Plant J **80**: 1085–1094

Hilker M, Fatouros NE (2015) Plant Responses to Insect Egg Deposition. Annu Rev Entomol **60**: 493–515

Hilker M, Kobs C, Varama M, Schrank K (2002) Induction of plant synomones by egg deposition. 7

Hilker M, Stein C, Schröder R, Varama M, Mumm R (2005) Insect egg deposition induces defence responses in Pinus sylvestris: characterisation of the elicitor. Journal of Experimental Biology **208**: 1849–1854

Holmes EC, Chen Y-C, Mudgett MB, Sattely ES (2021) *Arabidopsis* UGT76B1 glycosylates *N* -hydroxy-pipecolic acid and inactivates systemic acquired resistance in tomato. The Plant Cell. doi: 10.1093/plcell/koaa052

Howe GA, Jander G (2008) Plant Immunity to Insect Herbivores. Annu Rev Plant Biol **59**: 41–66

Ingle RA, Stoker C, Stone W, Adams N, Smith R, Grant M, Carré I, Roden LC, Denby KJ (2015) Jasmonate signalling drives time-of-day differences in susceptibility of Arabidopsis to the fungal pathogen *Botrytis cinerea*. Plant J **84**: 937–948

Jirage D, Tootle TL, Reuber TL, Frost LN, Feys BJ, Parker JE, Ausubel FM, Glazebrook J (1999) Arabidopsis thaliana PAD4 encodes a lipase-like gene that is important for salicylic acid signaling. Proceedings of the National Academy of Sciences **96**: 13583–13588

Jones JDG, Dangl JL (2006) The plant immune system. Nature **444**: 323–329

van Kan JAL (2006) Licensed to kill: the lifestyle of a necrotrophic plant pathogen. Trends in Plant Science **11**: 247–253

Kettles GJ, Drurey C, Schoonbeek H, Maule AJ, Hogenhout SA (2013) Resistance of *A rabidopsis thaliana* to the green peach aphid, *M yzus persicae* , involves camalexin and is regulated by micro RNA s. New Phytol **198**: 1178–1190

Little D, Gouhier-Darimont C, Bruessow F, Reymond P (2007) Oviposition by Pierid Butterflies Triggers Defense Responses in Arabidopsis. Plant Physiol **143**: 784–800

Liu Y, Sun T, Sun Y, Zhang Y, Radojičić A, Ding Y, Tian H, Huang X, Lan J, Chen S, et al (2020) Diverse Roles of the Salicylic Acid Receptors NPR1 and NPR3/NPR4 in Plant Immunity. Plant Cell **32**: 4002–4016

Lortzing T, Kunze R, Steppuhn A, Hilker M, Lortzing V (2020) Arabidopsis, tobacco, nightshade and elm take insect eggs as herbivore alarm and show similar transcriptomic alarm responses. Sci Rep **10**: 16281

Lortzing V, Oberländer J, Lortzing T, Tohge T, Steppuhn A, Kunze R, Hilker M (2019) Insect egg deposition renders plant defence against hatching larvae more effective in a salicylic acid-dependent manner. 14

Malamy J, Carr JP, Klessig DF, Raskin I (1990) Salicylic Acid: A Likely Endogenous Signal in the Resistance Response of Tobacco to Viral Infection. Science **250**: 1002–1004

Mäntylä E, Kleier S, Lindstedt C, Kipper S, Hilker M (2018) Insectivorous Birds Are Attracted by Plant Traits Induced by Insect Egg Deposition. J Chem Ecol **44**: 1127–1138

Mao G, Meng X, Liu Y, Zheng Z, Chen Z, Zhang S (2011) Phosphorylation of a WRKY Transcription Factor by Two Pathogen-Responsive MAPKs Drives Phytoalexin Biosynthesis in *Arabidopsis*. Plant Cell **23**: 1639–1653

Meehan TD, Werling BP, Landis DA, Gratton C (2011) Agricultural landscape simplification and insecticide use in the Midwestern United States. Proceedings of the National Academy of Sciences **108**: 11500–11505

Metraux JP, Signer H, Ryals J, Ward E, Wyss-Benz M, Gaudin J, Raschdorf K, Schmid E, Blum W, Inverardi B (1990) Increase in Salicylic Acid at the Onset of Systemic Acquired Resistance in Cucumber. Science **250**: 1004–1006

Mishina TE, Zeier J (2006) The Arabidopsis Flavin-Dependent Monooxygenase FMO1 Is an Essential Component of Biologically Induced Systemic Acquired Resistance. Plant Physiol **141**: 1666–1675

Mohnike L, Rekhter D, Huang W, Feussner K, Tian H, Herrfurth C, Zhang Y, Feussner I (2021) The glycosyltransferase UGT76B1 modulates N-hydroxy-pipecolic acid homeostasis and plant immunity. The Plant Cell **0**: 15

Mrazova A, Sam K, Amo L (2019) What do we know about birds' use of plant volatile cues in tritrophic interactions? Current Opinion in Insect Science **32**: 131–136

Mumm R, Schrank K, Wegener R, Schulz S, Hilker M (2003) CHEMICAL ANALYSIS OF VOLATILES EMITTED BY Pinus. Journal of Chemical Ecology 18

Nallu S, Hill JA, Don K, Sahagun C, Zhang W, Meslin C, Snell-Rood E, Clark NL, Morehouse NI, Bergelson J, et al (2018) The molecular genetic basis of herbivory between butterflies and their host plants. Nat Ecol Evol **2**: 1418–1427

Návarová H, Bernsdorff F, Döring A-C, Zeier J (2012) Pipecolic Acid, an Endogenous Mediator of Defense Amplification and Priming, Is a Critical Regulator of Inducible Plant Immunity. Plant Cell **24**: 5123–5141

Nawrath C, Métraux J-P (1999) Salicylic Acid Induction–Deficient Mutants of Arabidopsis Express PR-2 and PR-5 and Accumulate High Levels of Camalexin after Pathogen Inoculation. 13

Ngou BPM, Ahn H-K, Ding P, Jones JDG (2021) Mutual potentiation of plant immunity by cell-surface and intracellular receptors. Nature. doi: 10.1038/s41586-021-03315-7

Noutoshi Y, Okazaki M, Kida T, Nishina Y, Morishita Y, Ogawa T, Suzuki H, Shibata D, Jikumaru Y, Hanada A, et al (2012) Novel Plant Immune-Priming Compounds Identified via High-Throughput Chemical Screening Target Salicylic Acid Glucosyltransferases in *Arabidopsis*. Plant Cell **24**: 3795–3804

Orlovskis Z, Reymond P (2020) *Pieris brassicae* eggs trigger interplant systemic acquired resistance against a foliar pathogen in Arabidopsis. New Phytol **228**: 1652–1661

Pashalidou FG, Lucas-Barbosa D, van Loon JJA, Dicke M, Fatouros NE (2013) Phenotypic plasticity of plant response to herbivore eggs: effects on resistance to caterpillars and plant development. Ecology **94**: 702–713

Pauwels L, Barbero GF, Geerinck J, Tilleman S, Grunewald W, Pérez AC, Chico JM, Bossche RV, Sewell J, Gil E, et al (2010) NINJA connects the co-repressor TOPLESS to jasmonate signalling. Nature **464**: 788–791

Peng Y, Yang J, Li X, Zhang Y (2021) Salicylic Acid: Biosynthesis and Signaling. Annu Rev Plant Biol **72**: annurev-arplant-081320-092855

Petzold-Maxwell J, Wong S, Arellano C, Gould F (2011) Host plant direct defence against eggs of its specialist herbivore, Heliothis subflexa. Ecological Entomology **36**: 700–708

Piasecka A, Jedrzejczak-Rey N, Bednarek P (2015) Secondary metabolites in plant innate immunity: conserved function of divergent chemicals. New Phytol **206**: 948–964

Pieterse CMJ, Leon-Reyes A, Van der Ent S, Van Wees SCM (2009) Networking by small-molecule hormones in plant immunity. Nat Chem Biol **5**: 308–316

Pieterse CMJ, Zamioudis C, Berendsen RL, Weller DM, Van Wees SCM, Bakker PAHM (2014) Induced Systemic Resistance by Beneficial Microbes. Annu Rev Phytopathol **52**: 347–375

Rajniak J, Barco B, Clay NK, Sattely ES (2015) A new cyanogenic metabolite in Arabidopsis required for inducible pathogen defence. Nature **525**: 376–379

Ranf S (2017) Sensing of molecular patterns through cell surface immune receptors. Current Opinion in Plant Biology **38**: 68–77

Rekhter D, Lüdke D, Ding Y, Feussner K, Zienkiewicz K, Lipka V, Wiermer M, Zhang Y, Feussner I (2019) Isochorismate-derived biosynthesis of the plant stress hormone salicylic acid. Science **365**: 498–502

Reymond P (2013) Perception, signaling and molecular basis of oviposition-mediated plant responses. Planta **238**: 247–258

Reymond P, Bodenhausen N, Van Poecke RMP, Krishnamurthy V, Dicke M, Farmer EE (2004) A Conserved Transcript Pattern in Response to a Specialist and a Generalist Herbivore. Plant Cell **16**: 3132–3147

Ross AF (1961) Systemic acquired resistance induced by localized virus infections in plants. Virology **14**: 340–358

Roux M, Schwessinger B, Albrecht C, Chinchilla D, Jones A, Holton N, Malinovsky FG, Tör M, de Vries S, Zipfel C (2011) The *Arabidopsis* Leucine-Rich Repeat Receptor–Like Kinases BAK1/SERK3 and BKK1/SERK4 Are Required for Innate Immunity to Hemibiotrophic and Biotrophic Pathogens. Plant Cell **23**: 2440–2455

von Saint Paul V, Zhang W, Kanawati B, Geist B, Faus-Keßler T, Schmitt-Kopplin P, Schäffner AR (2011) The *Arabidopsis* Glucosyltransferase UGT76B1 Conjugates Isoleucic Acid and Modulates Plant Defense and Senescence. Plant Cell **23**: 4124–4145

Schweizer F, Fernández-Calvo P, Zander M, Diez-Diaz M, Fonseca S, Glauser G, Lewsey MG, Ecker JR, Solano R, Reymond P (2013) *Arabidopsis* Basic Helix-Loop-Helix Transcription Factors MYC2, MYC3, and MYC4 Regulate Glucosinolate Biosynthesis, Insect Performance, and Feeding Behavior. Plant Cell **25**: 3117–3132

Sels J, Mathys J, De Coninck BMA, Cammue BPA, De Bolle MFC (2008) Plant pathogenesis-related (PR) proteins: A focus on PR peptides. Plant Physiology and Biochemistry **46**: 941–950

Shah J, Zeier J (2013) Long-distance communication and signal amplification in systemic acquired resistance. Front Plant Sci. doi: 10.3389/fpls.2013.00030

Shapiro AM, DeVay JE (1987) Hypersensitivity reaction of Brassica nigra L. (Crueiferae) kills eggs of Pieris butterflies (Lepidoptera: Pieridae). 2

Sharma M, Bhatt D (2015) The circadian clock and defence signalling in plants: Clock-defence cross-talk. Molecular Plant Pathology **16**: 210–218

Stahl E, Bellwon P, Huber S, Schlaeppi K, Bernsdorff F, Vallat-Michel A, Mauch F, Zeier J (2016) Regulatory and Functional Aspects of Indolic Metabolism in Plant Systemic Acquired Resistance. Molecular Plant **9**: 662–681

Stahl E, Brillatz T, Ferreira Queiroz E, Marcourt L, Schmiesing A, Hilfiker O, Riezman I, Riezman H, Wolfender J-L, Reymond P (2020) Phosphatidylcholines from Pieris brassicae eggs activate an immune response in Arabidopsis. eLife **9**: e60293

Stahl E, Hilfiker O, Reymond P (2018) Plant-arthropod interactions: who is the winner? Plant J **93**: 703–728

Sun JY, Sønderby IE, Halkier BA, Jander G, de Vos M (2009) Non-Volatile Intact Indole Glucosinolates are Host Recognition Cues for Ovipositing Plutella xylostella. J Chem Ecol **35**: 1427–1436

Sun T, Busta L, Zhang Q, Ding P, Jetter R, Zhang Y (2018) TGACG-BINDING FACTOR 1 (TGA1) and TGA4 regulate salicylic acid and pipecolic acid biosynthesis by modulating the expression of *SYSTEMIC ACQUIRED RESISTANCE DEFICIENT 1* (*SARD1*) and *CALMODULIN-BINDING PROTEIN 60g* (*CBP60g*). New Phytol **217**: 344–354

Sun T, Zhang Y, Li Y, Zhang Q, Ding Y, Zhang Y (2015) ChIP-seq reveals broad roles of SARD1 and CBP60g in regulating plant immunity. Nat Commun **6**: 10159

Thies C, Steffan-Dewenter I, Tscharntke T (2003) Effects of landscape context on herbivory and parasitism at different spatial scales. Oikos **101**: 18–25

Thines B, Katsir L, Melotto M, Niu Y, Mandaokar A, Liu G, Nomura K, He SY, Howe GA, Browse J (2007) JAZ repressor proteins are targets of the SCFCOI1 complex during jasmonate signalling. Nature **448**: 661–665

Thomma BPHJ, Eggermont K, Tierens KFM-J, Broekaert WF (1999) Requirement of Functional *Ethylene-Insensitive 2* Gene for Efficient Resistance of Arabidopsis to Infection by *Botrytis cinerea*. Plant Physiol **121**: 1093–1101

Torrens-Spence MP, Bobokalonova A, Carballo V, Glinkerman CM, Pluskal T, Shen A, Weng J-K (2019) PBS3 and EPS1 Complete Salicylic Acid Biosynthesis from Isochorismate in Arabidopsis. Molecular Plant **12**: 1577–1586

Valsamakis G, Bittner N, Fatouros NE, Kunze R, Hilker M, Lortzing V (2020) Priming by Timing: Arabidopsis thaliana Adjusts Its Priming Response to Lepidoptera Eggs to the Time of Larval Hatching. Front Plant Sci **11**: 619589

Veloso J, van Kan JAL (2018) Many Shades of Grey in Botrytis–Host Plant Interactions. Trends in Plant Science **23**: 613–622

Vernooij B, Reist LFM, KolditzJawhar R, Ward E, Uknes S, Kessmann H, Ryals J (1994) Salicylic Acid 1s Not the Translocated Signal Responsible for lnducing Systemic Acquired Resistance but 1s Required in Signal Transduction. 8

Vlot AC, Dempsey DA, Klessig DF (2009) Salicylic Acid, a Multifaceted Hormone to Combat Disease. Annu Rev Phytopathol **47**: 177–206

Vlot AC, Sales JH, Lenk M, Bauer K, Brambilla A, Sommer A, Chen Y, Wenig M, Nayem S (2020) Systemic propagation of immunity in plants. New Phytologist 17

Wang L, Tsuda K, Truman W, Sato M, Nguyen LV, Katagiri F, Glazebrook J (2011) CBP60g and SARD1 play partially redundant critical roles in salicylic acid signaling: Role of CBP60 proteins in salicylic acid signaling. The Plant Journal **67**: 1029–1041

Wang M, Weiberg A, Lin F-M, Thomma BPHJ, Huang H-D, Jin H (2016) Bidirectional cross-kingdom RNAi and fungal uptake of external RNAs confer plant protection. Nature Plants **2**: 16151

Wang Y, Schuck S, Wu J, Yang P, Döring A-C, Zeier J, Tsuda K (2018) A MPK3/6-WRKY33-ALD1-Pipecolic Acid Regulatory Loop Contributes to Systemic Acquired Resistance. Plant Cell **30**: 2480–2494

Wegener R, Schulz S, Meiners T, Hilker M (2001) Analysis of Volatiles Induced by Oviposition of Elm Leaf Beetle Xanthogaleruca luteola on Ulmus minor. 17

Weiberg A, Wang M, Lin F-M, Zhao H, Zhang Z, Kaloshian I, Huang H-D, Jin H (2013) Fungal Small RNAs Suppress Plant Immunity by Hijacking Host RNA Interference Pathways. Science **342**: 118–123

Wildermuth MC, Dewdney J, Wu G, Ausubel FM (2001) Isochorismate synthase is required to synthesize salicylic acid for plant defence. Nature **414**: 562–565

Williams KS, Gilbert LE (1981) Insects as Selective Agents on Plant Vegetative Morphology: Egg Mimicry Reduces Egg Laying by Butterflies. Science **212**: 467–469

Williamson B, Tudzynski B, Tudzynski P, Van Kan JAL (2007) Botrytis cinerea: the cause of grey mould disease. Mol Plant Pathol **8**: 561–580

Wittstock U, Agerbirk N, Stauber EJ, Olsen CE, Hippler M, Mitchell-Olds T, Gershenzon J, Vogel H (2004) Successful herbivore attack due to metabolic diversion of a plant chemical defense. Proceedings of the National Academy of Sciences **101**: 4859–4864

Yang J-O, Nakayama N, Toda K, Tebayashi S, Kim C-S (2014) Structural determination of elicitors in *Sogatella furcifera* (Horváth) that induce Japonica rice plant varieties (*Oryza sativa* L.) to produce an ovicidal substance against *S. furcifera* eggs. Bioscience, Biotechnology, and Biochemistry **78**: 937–942

Yu X, Feng B, He P, Shan L (2017) From Chaos to Harmony: Responses and Signaling upon Microbial Pattern Recognition. Annu Rev Phytopathol **55**: 109–137

Yuan M, Jiang Z, Bi G, Nomura K, Liu M, Wang Y, Cai B, Zhou J-M, He SY, Xin X-F (2021) Pattern-recognition receptors are required for NLR-mediated plant immunity. Nature. doi: 10.1038/s41586-021-03316-6

Zeier J (2021) Metabolic regulation of systemic acquired resistance. Current Opinion in Plant Biology **62**: 102050

Zeier J (2013) New insights into the regulation of plant immunity by amino acid metabolic pathways: Amino acid metabolism and plant immunity. Plant Cell Environ **36**: 2085–2103

Zhang W, Corwin JA, Copeland D, Feusier J, Eshbaugh R, Chen F, Atwell S, Kliebenstein DJ (2017) Plastic Transcriptomes Stabilize Immunity to Pathogen Diversity: The Jasmonic Acid and Salicylic Acid Networks within the Arabidopsis/ *Botrytis* Pathosystem. Plant Cell **29**: 2727–2752

Zhang Y, Xu S, Ding P, Wang D, Cheng YT, He J, Gao M, Xu F, Li Y, Zhu Z, et al (2010) Control of salicylic acid synthesis and systemic acquired resistance by two members of a plant-specific family of transcription factors. Proceedings of the National Academy of Sciences **107**: 18220–18225

Zhao Y, Hull AK, Gupta NR, Goss KA, Alonso J, Ecker JR, Normanly J, Chory J, Celenza JL (2002) Trp-dependent auxin biosynthesis in Arabidopsis: involvement of cytochrome P450s CYP79B2 and CYP79B3. 13

Zhou J, Wang X, He Y, Sang T, Wang P, Dai S, Zhang S, Meng X (2020) Differential Phosphorylation of the Transcription Factor WRKY33 by the Protein Kinases CPK5/CPK6 and MPK3/MPK6 Cooperatively Regulates Camalexin Biosynthesis in Arabidopsis. Plant Cell **32**: 2621–2638

Zhou J-M, Zhang Y (2020) Plant Immunity: Danger Perception and Signaling. Cell **181**: 978–989

CHAPTER 1

Insect eggs induce a systemic acquired resistance against *Botrytis cinerea* in *Arabidopsis*

Esteban Alfonso, Elia Stahl, Etienne Bellani and Philippe Reymond

Department of Plant Molecular Biology, University of Lausanne, Switzerland

ABSTRACT

Although insect eggs look harmless, they constitute a real threat for the plant as they will develop into feeding larvae and cause important damages. In *Arabidopsis thaliana*, oviposition by the Large White butterfly *Pieris brassicae* induces expression of defence genes associated to biotic stresses and accumulation of salicylic acid (SA), which is usually produced to fend off bacterial pathogens rather than herbivorous insects. Previous work showed that natural oviposition by *P. brassicae* or treatment with egg extract (EE) trigger a systemic acquired resistance (SAR) against the hemi-biotroph plant pathogen *Pseudomonas syringae*. Here, we found that EE-induced SAR is also effective against the fungal pathogen *Botrytis cinerea*. Application of EE from the generalist *Spodoptera littoralis* as well as insect egg-associated phosphatidylcholines (PCs) also induced a significant reduction of *B. cinerea* growth in systemic leaves. This EE-induced SAR is dependent on the lectin receptor kinase LecRK-I.8 and on mitogen-activated protein kinases MPK3 and MPK6, which are involved in early egg signalling steps. Furthermore, EE-induced SAR is abolished in *ics1*, *ald1* and *fmo1*, indicating that functional SA and *N*-hydroxy-pipecolic acid (NHP) pathways are required. Together, these results show that *P. brassicae* oviposition induces SAR against pathogens with different lifestyles through a conserved signalling mechanism.

INTRODUCTION

Due to their inability to escape enemies, plants have evolved strategies to face a broad range of attackers. These strategies mainly rely on transcriptional changes mediated by jasmonic acid (JA), salicylic acid (SA) and ethylene (ET) signalling pathways (Bürger and Chory, 2019). Upon recognition of herbivory, plants activate the JA pathway, which leads to the production of poisonous metabolites and anti-digestive proteins as well as the emission of volatiles that attract predators (Reymond et al., 2004; Howe and Jander, 2008).

Although insect eggs deposited on leaves do not represent a direct threat, they trigger various direct and indirect defence responses that lead to egg desiccation, drop-off, mortality or attraction of egg parasitoids (Reymond, 2013; Hilker and Fatouros, 2016). Following oviposition by the Large White butterfly *Pieris brassicae*, the expression profile of *Arabidopsis thaliana* (*Arabidopsis*) leaves is drastically distinct from the profile induced after larval feeding (Little et al., 2007). Indeed, oviposition-induced expression profile is enriched with SA-regulated genes (Little et al., 2007). Accordingly, SA accumulates at the site of oviposition as well as in systemic tissues (Bruessow et al., 2010). Egg-induced SA accumulation was shown to inhibit the JA pathway, resulting in an enhanced performance of the generalist *Spodoptera littoralis* larvae (Bruessow et al., 2010), although this effect is variable across plants and insects species (Bandoly et al., 2015; Bonnet et al., 2017; Lortzing et al., 2019). Furthermore, many components of the SA pathway, such as the downstream regulator NON-EXPRESSOR OF PR GENES 1 (NPR1) are important for egg-induced gene expression (Gouhier-Darimont et al., 2013). In addition to SA, *P. brassicae* oviposition and treatment with crude egg extract (EE) induce the accumulation of reactive oxygen species (ROS) and trigger a localized cell death, which are responses usually produced to fend off pathogens during pattern-triggered immunity (PTI) (Gouhier-Darimont et al., 2013). In plants, lectins have been described as important actors in defence against pathogens (Chrispeels and Raikhelb, 1991). Interestingly, the L-type lectin receptor kinase LecRK-I.8 was recently shown to be involved in insect egg perception (Gouhier-Darimont et al., 2019). Indeed, the *lecrk-I.8* mutant displays drastically reduced responses following *P. brassicae* oviposition and treatment with EE (Gouhier-Darimont et al., 2013; Gouhier-Darimont et al., 2019). Recently, phosphatidylcholines (PCs) were shown to be the most abundant lipid species in *P. brassicae* and *S. littoralis* eggs (Stahl et al., 2020). PCs can diffuse out of the eggs and induce SA accumulation, cell death, hydrogen peroxide production and defence gene expression in *Arabidopsis*, similar to natural oviposition and

treatment with EE and function as active egg-associated molecular patterns (EAMPs) (Stahl et al., 2020).

It was recently shown that *P. brassicae* oviposition and treatment with EE induce a systemic acquired resistance (SAR) in *Arabidopsis* (Hilfiker et al., 2014). SAR is an inducible defence response commonly associated with a primary pathogen infection which results in enhanced systemic defences and protection upon a secondary infection by a broad range of pathogens (Fu and Dong, 2013; Vlot et al., 2020). Plants pre-treated with EE are more resistant to the bacterial pathogen *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*) compared to untreated plants and this is associated with the priming of defence gene expression (Hilfiker et al., 2014). SAR requires the SA pathway and the translocation of a mobile signal (Shah and Zeier, 2013; Vlot et al., 2020). Recently, the non-protein amino acid pipecolic acid (Pip) derivative *N*-hydroxy-Pip (NHP) was identified as an active SAR signal (Návarová et al., 2012; Chen et al., 2018; Hartmann et al., 2018). Indeed, Pip and NHP accumulate in local and systemic leaves following infection by *P. syringae* pv. *maculicola* (*Psm*) and promote SA accumulation as well as defence gene expression (Návarová et al., 2012; Hartmann et al., 2018). The first step of NHP biosynthesis is catalysed by AGD2-LIKE DEFENSE RESPONSE PROTEIN 1 (ALD1) which transfers the amino group of L-lysine to generate 2,3-dehydropipecolic acid, which is then reduced to Pip by SAR-DEFICIENT 4 (SARD4) (Hartmann et al., 2017). FLAVIN-DEPENDENT MONOOXYGENASE 1 (FMO1) further converts Pip to NHP, which acts as the metabolic SAR regulator (Chen et al., 2018; Hartmann et al., 2018). Consequently, pathogen-induced SAR is abolished in *ald1* and *fmo1* mutants (Návarová et al., 2012; Ding et al., 2016; Hartmann et al., 2017). However, when exogenously applied, Pip can restore SAR in *ald1* but not in *fmo1* mutant, whereas application of NHP restores SAR in *fmo1*, indicating that NHP is the active SAR signal (Návarová et al., 2012; Chen et al., 2018). Interestingly, *P. brassicae* EE treatment induces Pip accumulation in local and distal leaves and EE-induced SAR is also blocked in *ald1* and *fmo1*, implicating the NHP pathway in this response and illustrating a conserved mechanism with bacterial-induced SAR (Hilfiker et al., 2014).

In this study, we show that egg-induced SAR is also efficient against the fungal pathogen *Botrytis cinerea*. This generalist pathogen causes the grey mould disease and infects more than 1000 plant species (Veloso and van Kan, 2018). Here, we focus on the signalling of egg-induced SAR against *B. cinerea*. We show that this response requires early-egg perception components and is dependent on the SA and NHP pathways.

RESULTS

Oviposition and treatment with egg extract reduce *B. cinerea* infection

It was previously found that natural oviposition and treatment with *P. brassicae* EE reduce growth of several *P. syringae* strains in *Arabidopsis*, both in local and distal leaves (Hilfiker et al., 2014). Here, we tested whether insect eggs can induce a similar response to *B. cinerea* infection. For that purpose, female *P. brassicae* butterflies were allowed to deposit egg batches on 4-week-old *Arabidopsis* plants for 4 to 5 days. Just before hatching of larvae, eggs were gently removed from the plant. Two distal leaves were then infected by drop inoculation with a *B. cinerea* spore suspension and lesion perimeter was measured after 3 days (Fig. 1A). Compared to control plants, oviposited plants showed a significantly reduced infection in distal leaves (Fig. 1B). As a complementary experiment, plants were pretreated with *P. brassicae* EE for 5 days and then two distal leaves were infected. The amount of EE applied onto each plant was equivalent to approximatively two egg batches (one batch per leaf), consisting of 20-30 eggs each. A similar reduction of *B. cinerea* infection was observed in EE-treated plants, compared to control plants (Fig. 1C). These results confirm previous observations that EE treatment mimics responses triggered by natural oviposition (Little et al., 2007; Bruessow et al., 2010; Stahl et al., 2020). All further experiments were thus carried out using EE instead of natural egg deposition. Consistent with observations on lesion size, hyphal development was also significantly reduced in distal leaves (Supplemental Fig. 1). In addition, expression of *B. cinerea* β-tubulin gene was significantly lower in EE-treated plants (Fig. 1D), providing independent confirmation that EE pretreatment reduces *B. cinerea* infection. Finally, a time-course experiment in local and distal leaves showed that inhibition of *B. cinerea* infection can be observed from 48 h to 72 h, with a significant reduction in distal leaves only after 72 h (Supplemental Fig. 2). To explore the generality of egg-induced responses, plants were pretreated with EE from the generalist herbivore *Spodoptera littoralis*. Similar to *P. brassicae*, pretreatment with *S. littoralis* EE significantly reduced *B. cinerea* infection (Fig. 1E), suggesting conserved signalling mechanisms between distantly related insect species. Application of purified PCs onto *Arabidopsis* leaves was recently shown to induce similar immune responses than EE application and natural oviposition (Stahl et al., 2020). To investigate whether PCs induce a similar response than EE to *B. cinerea* infection, plants were pretreated with a PC-Mix solution for 5 days and distal leaves were infected. Similar to EE

pretreatment, application of PCs significantly reduced *B. cinerea* infection in distal leaves (Fig. 1F) and in local leaves (Supplemental Fig. 3).

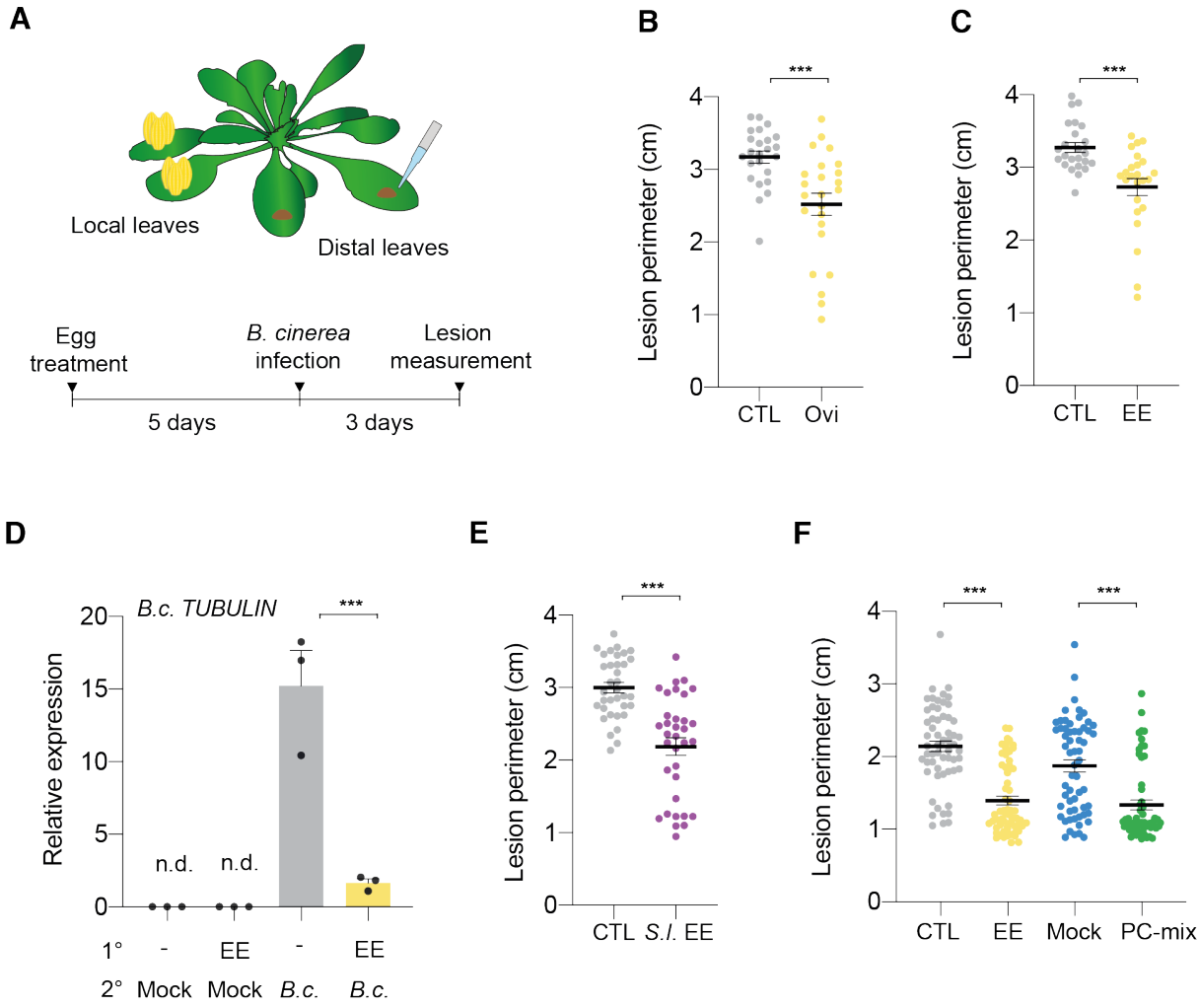


Figure 1. Oviposition and treatment with EE reduce *Botrytis cinerea* infection.
(**A**) Experimental design. (**B**) Effect of 5 days-pretreatment with *P. brassicae* oviposition (Ovi) on *B. cinerea* lesion perimeter in distal leaves was measured 3 days after inoculation. Inoculated plants without pretreatment were used as controls (CTL). (**C**) Same as **B** but plants were pretreated with *P. brassicae* egg extract (EE). (**D**) Expression of the *B. cinerea* tubulin gene in distal leaves. Local leaves (1°) were either treated with *P. brassicae* EE (EE) for 5 days or not treated (-). Distal leaves (2°) were then inoculated with PDB (Mock) or *B. cinerea* spore suspension (*B.c.*) for 2 days. "n.d.": non-detectable expression. (**E**) Same as **C** but plants were pretreated with *S. littoralis* EE. (**F**) Same as **C** but plants were pretreated with either *P. brassicae* EE or a solution of PC-mix from chicken egg. Respective controls consisted of untreated plants (CTL) or plants treated with a mock solution (Mock). Values shown in (**B**, **C**, **D**, **E** and **F**) are means ± SE of three independent experiments (n = 8-20 leaves per experiment). Significant differences between control and treated plants are indicated (linear mixed model, *** $P<0.001$). Dots indicate individual values.

EE-induced SAR requires early egg perception signalling

Recently, it was found that EE-induced SAR against *Pst* was abolished in the *lecrk-I.8* single mutant (Gouhier-Darimont et al., 2019). To investigate whether LecRK-I.8 is involved in EE-induced SAR against *B. cinerea*, *lecrk-I.8* mutant was pretreated with *P. brassicae* EE for 5 days and two distal leaves were infected. After 3 days, lesion sizes were measured and compared to control plants. Pretreatment with EE significantly reduced *B. cinerea* infection in distal leaves and this effect was completely abolished in the *lecrk-I.8* mutant (Fig. 2A), suggesting a conserved mechanism with EE-induced SAR against *Pst*.

Following PAMPs recognition, downstream signalling events are controlled by the redundant mitogen-activated protein kinases (MPK) MPK3 and MPK6, which have been shown to also play a role in SAR establishment (Beckers et al., 2009; Wang et al., 2018). To assess the involvement of these kinases in EE-induced SAR, *mpk3-1* and *mpk6-2* mutants were pretreated with EE and then infected with *B. cinerea*. In both mutants, EE-induced reduction of *B. cinerea* growth was abolished (Fig. 2B), suggesting a requirement of these components for EE-induced systemic responses.

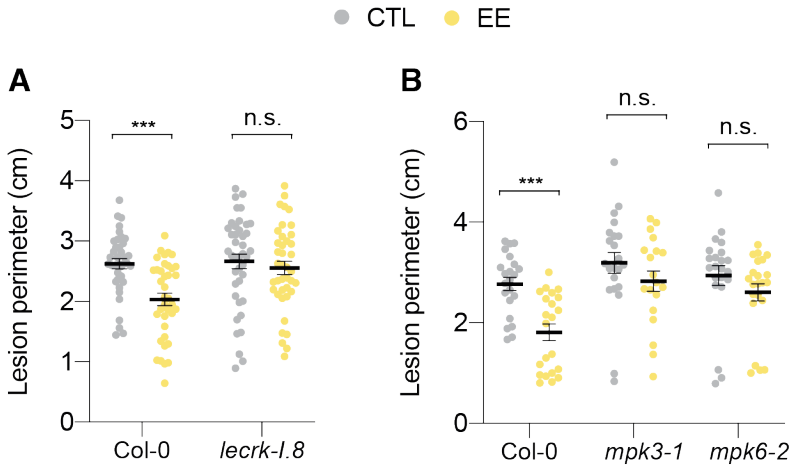


Figure 2. EE-induced SAR requires early egg perception signalling.
(**A**, **B**) Plant genotypes were pretreated with *P. brassicae* EE for 5 days and further infected with *B. cinerea* for 3 days. Lesion perimeter was measured in control (CTL) and distal leaves from EE-treated plants (EE). Means ± SE of three independent experiments are shown (n = 8-14 leaves per experiment). Significant differences between CTL and treatment are indicated (linear mixed model, *** $P<0.001$; n.s., not significant). Dots indicate individual values.

EE-induced SAR depends on the SA pathway

SAR establishment relies on the SA pathway (Hartmann and Zeier, 2019), which is activated in *Arabidopsis* by *P. brassicae* oviposition (Little et al., 2007; Bruessow et al., 2010). SA-deficient mutants, such as *ics1* or SA signalling mutants, such as *npr1-1* exhibit altered responses to *P. brassicae* oviposition (Gouhier-Darimont et al., 2013). We used several mutants impaired in either SA biosynthesis or SA signalling to test the implication of the SA pathway in EE-induced SAR against *B. cinerea*. SA biosynthesis requires primarily the activity of ISOCHORISMATE SYNTHASE 1 (ICS1), with a limited contribution of its homolog ICS2 (Garcion et al., 2008). Contrary to Col-0, EE-induced SAR was abolished in *ics1* and *ics1*$^{-/-}$ *ics2*$^{-/+}$ but conserved in *ics2* (Fig. 3A). The *ics1*$^{-/-}$ *ics2*$^{-/+}$ double mutant is homozygous ($^{-/-}$) for the mutation in *ICS1* but heterozygous ($^{-/+}$) for the mutation in *ICS2* gene. The fully homozygous *ics1 ics2* double mutant is severely impacted in growth and leaves pigmentation (pale yellowish leaves) (Garcion et al., 2008). We thus decided not to use it for this work. Consistent with mutants impaired in SA biosynthesis, the SA-degrading transgenic plant *nahG* displayed no EE-induced SAR but a slight increase in susceptibility to *B. cinerea* following EE pre-treatment (Fig. 3A). Following five days of EE treatment, Col-0 and *ics2* mutant accumulated SA levels in EE-treated leaves, whereas the EE-induced SAR-defective mutants *ics1, ics1*$^{-/-}$ *ics2*$^{-/+}$ and *nahG* did not (Fig. 3B). These results strongly suggest that EE-induced SAR establishment relies on the induction of SA accumulation in local leaves following EE treatment (Fig. 3B).

In PTI signalling, NON EXPRESSOR OF PR GENES 1 (NPR1) and NPR3/NPR4 are important downstream modulators of defence gene expression (Zhou and Zhang, 2020). They all bind to SA but NPR1 acts as a positive activator of transcription, whereas NPR3/NPR4 are repressors (Zhou and Zhang, 2020). We previously found that EE-induced *PR1* expression was significantly reduced in *npr1-1* (Gouhier-Darimont et al., 2013). Here, *npr1-1* displayed a reduced EE-induced SAR, although this response was not completely abolished (Fig. 3C). The residual signalling activity in *npr1-1* is postulated to be due to the inhibition of NPR3/NPR4 repressor activity by SA (Liu et al., 2020). Indeed, using the *npr1-1 npr4-4D* double mutant, which includes the gain-of-function mutant *npr4-4D* and which is blocked in SA signalling (Liu et al., 2020), we could not detect any SAR (Fig. 3D). The double mutant was also more susceptible to *B. cinerea* in absence of EE pretreatment. Thus, these findings demonstrate a contribution of the two groups of SA receptors in basal resistance and EE-induced SAR against *B. cinerea*.

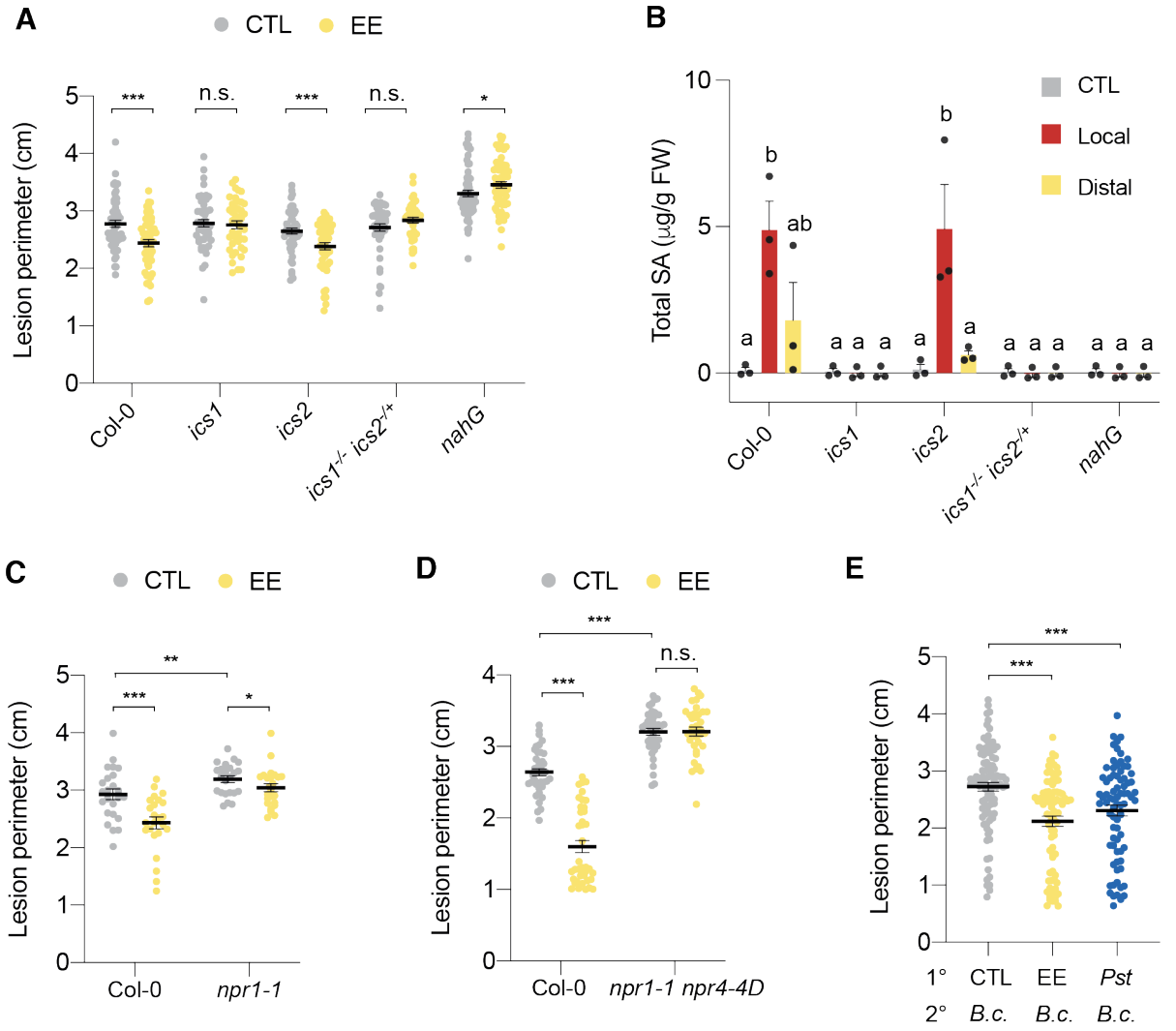


Figure 3. EE-induced SAR depends on the SA pathway.
(**A**, **C** and **D**) Plant genotypes were pretreated with *P. brassicae* EE for 5 days and further infected with *B. cinerea* for 3 days. Lesion perimeter was measured in control (CTL) and distal leaves from EE-treated plants (EE). The double mutant *ics1 ics2* was homozygous for *ics1* ($^{-/-}$) and heterozygous for *ics2* ($^{-/+}$). (**B**) Quantification of total SA in untreated plants (CTL), EE-treated leaves (Local) and in leaves distal to EE-treated leaves (Distal) after 5 days. Means ± SE of three independent experiments are shown (n = 6 leaves per experiment). Different letters indicate significant difference between treatments within genotypes at $P<0.05$ (ANOVA followed by Tukey's Honest Significant Difference test). (**E**) Local leaves (1°) were untreated (CTL), treated with EE for 5 days (EE) or infiltrated with *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*) for 2 days. Distal leaves (2°) were then inoculated with *B. cinerea* spore suspension (*B.c.*) for 3 days before lesion perimeter measurement. For all experiments, means ± SE of three independent experiments are shown (n = 8-28 leaves per experiment). Significant differences between CTL and treatment are indicated (linear mixed model, *** $P<0.001$; ** $P<0.01$; * $P<0.05$; n.s., not significant). Dots indicate individual values.

Finally, to test the involvement of other signalling pathways in this response, we used mutants impaired in ET signalling and JA biosynthesis. The ET-insensitive mutant *ein2-1* and the JA-deficient mutant *aos* were highly susceptible to *B. cinerea* infection and displayed no EE-induced SAR (Supplemental Fig. 4). Also, we tested whether another stimulus triggering

SA was able to induce SAR against *B. cinerea*. For that purpose, the effect of EE pre-treatment and local *Pst* inoculation on secondary infection with *B. cinerea* was compared simultaneously. Local leaves (1°) were either left untreated, pretreated with EE for 5 days or infiltrated with *Pst* for 2 days and distal leaves (2°) were infected with *B. cinerea* for 3 days. Both pretreatments triggered a significant and similar reduction of *B. cinerea* infection in distal leaves compared to untreated control plants (Fig. 3E). Together, these results indicate that EE-induced SAR against *B. cinerea* requires a fully functional SA pathway.

EE-induced SAR requires a functional NHP pathway

The lysine catabolite Pip and its derivative NHP are crucial for SAR establishment (Chen et al., 2018; Hartmann et al., 2018). It was recently shown that the NHP pathway is required for EE-induced SAR against bacterial pathogens (Hilfiker et al., 2014). Here, we used the Pip-devoid mutant *ald1* and the *fmo1* mutant, impaired in Pip conversion to NHP (Návarová et al., 2012; Hartmann et al., 2018). Both *ald1* and *fmo1* mutants were impaired in EE-induced SAR against *B. cinerea* (Fig. 4A), indicating that the NHP pathway is required to reduce fungal growth systemically. In addition, we tested whether exogenous Pip application could complement the SAR-defective phenotype of these mutants. Plants were left untreated or pretreated with EE for 5 days and one day prior *B. cinerea* infection, a 1 mM Pip solution was pipetted onto the soil of Col-0, *ald1* and *fmo1* plants for uptake via the root system (Návarová et al., 2012). In Col-0, Pip application alone did not increase resistance against *B. cinerea* and did not enhance EE-induced SAR (Fig. 4B), suggesting that Pip is not sufficient to induce SAR without an EE-derived stimulus. However, Pip application to *ald1* was able to restore SAR after EE treatment (Fig. 4B), indicating that Pip can complement the biosynthetic mutant and acts downstream of an EE stimulus. Finally, Pip application did not restore EE-induced SAR in *fmo1* (Fig. 4B).

Due to its crucial importance for SAR establishment, we assumed that SA might be the required EE-derived stimulus. To investigate this, we first infiltrated SA in different concentrations to leaves of Col-0 and *ics1*$^{-/-}$ *ics2*$^{-/+}$ in order to reach similar SA levels found in local EE-treated leaves. Infiltration of 0.25 mM SA allowed to reach ca. 10 µg/g FW of total SA in infiltrated (local) leaves, which is similar to levels reached after 5 days of EE treatment (Supplemental Fig. 5A, Fig. 3B). Infiltrated SA was not detected in distal leaves, suggesting that it does not move systemically (Supplemental Fig. 5A). Although infiltration of 0.5 mM SA efficiently induced *PR1* expression (Supplemental Fig. 5B), we used 0.25 mM SA for infiltration of Col-0, *ald1* and *fmo1* in an experiment attempting to reproduce SAR without EE

pretreatment. One day prior infection, plants were irrigated with a 1 mM Pip solution and 4 h before infection, two leaves were infiltrated with a 0.25 mM SA solution. Two distal leaves were then infected with *B. cinerea* and lesion sizes were measured 3 days later. SA infiltration in substitution of EE treatment did not trigger a SAR in any plant genotypes (Supplemental Fig. 5C). These results strongly suggest that NHP is the signal controlling EE-induced SAR against *B. cinerea* but that it requires another stimulus from eggs to initiate the response.

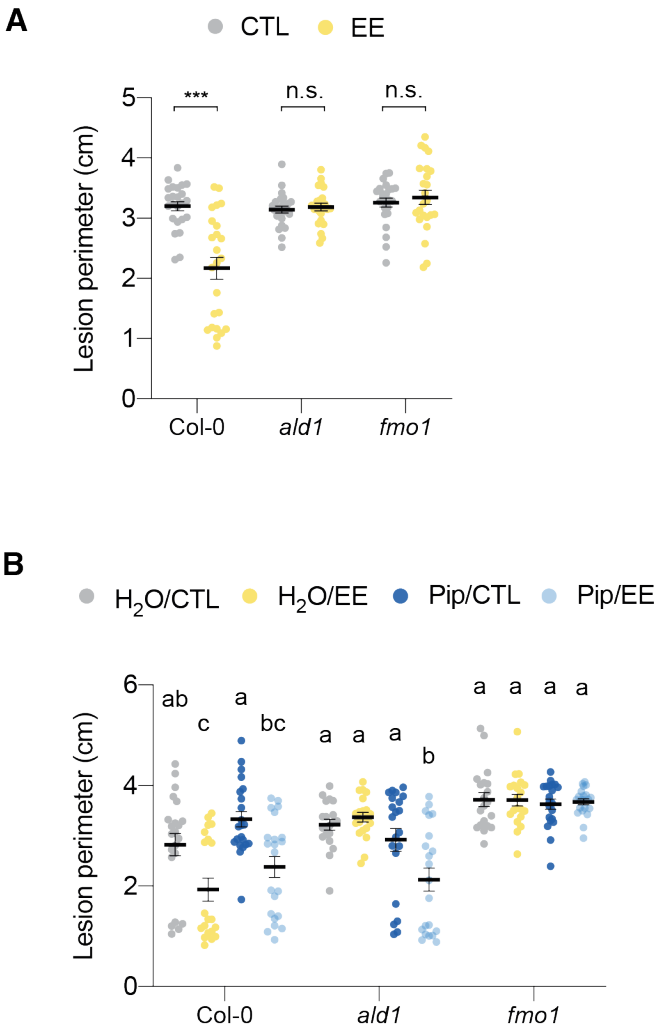


Figure 4. EE-induced SAR depends on the NHP pathway.
(**A**) Plant genotypes were pretreated with *P. brassicae* EE for 5 days and further infected with *B. cinerea* for 3 days. Lesion perimeter was measured in control (CTL) and distal leaves from EE-treated plants (EE). Means ± SE of three independent experiments are shown (n = 8-10 leaves per experiment). Significant differences between control and treated plants are indicated (linear mixed model, *** $P<0.001$; n.s., not significant). (**B**) Plant genotypes were pretreated with *P. brassicae* EE for 5 days and further infected with *B. cinerea*. H_2O or 1 mM pipecolic acid (Pip) was applied to the soil one day prior infection and lesion perimeter measurements were done 3 days after infection. Means ± SE of three independent experiments are shown (n = 6-8 leaves per experiment). Different letters indicate significant differences at $P<0.05$ (ANOVA followed by Tukey's Honest Significant Difference test). Dots indicate individual values.

DISCUSSION

In this study, we show that both natural oviposition and treatment with *P. brassicae* EE reduce *B. cinerea* infection in *Arabidopsis* through the activation of SAR. EE treatment induced protection in both local and distal leaves. Lesions were significantly smaller in local leaves 48 h after infection compared to distal leaves, suggesting that a faster protective response can occur locally. To reach distal leaves, a signal must be generated and translocated in the whole foliage, and this might take some time from its generation in local leaves to the actual effect in distal leaves. SA and NHP accumulation in distal leaves following local *Psm* infection peaks at 48 h post-infection (Hartmann and Zeier, 2019). However, whether SA and NHP simultaneously reach an active concentration following local EE application is unknown but if so, some additional time might be required for induction of systemic responses following perception of these signals. Also, EE might induce the accumulation of secondary metabolites with possible antifungal activity in local leaves, allowing a faster inhibition of *B. cinerea* growth. Interestingly, treatment with EE from the generalist *S. littoralis* also induced a SAR against *B. cinerea*. It was recently reported that egg-derived PCs can diffuse out and induce immune responses in *Arabidopsis*, thus acting as active EAMPs (Stahl et al., 2020). The PCs composition of EE from *P. brassicae* and *S. littoralis* is similar and this could explain the similarities between these EE-induced responses (Stahl et al., 2020). Furthermore, treatment with EEs from *P. brassicae*, *S. littoralis, Trichoplusia ni* and *Drosophila melanogaster* induces expression of the SA-marker gene *PR1* in *Arabidopsis* (Bruessow et al., 2010; Wang et al., 2017), suggesting the activation of a conserved signalling pathway between distinctly related insect species. Whether PCs concentration in the eggs of *T. ni* and *D. melanogaster* is similar to *P. brassicae* is unknown and would be interesting to quantify. Strikingly, application of purified PCs induced a similar reduction of *B. cinerea* infection in both local and distal leaves compared to *P. brassicae* EE. Plants respond actively to application of lipids by inducing immune responses. For example, extracts from the female planthopper *Sogatella furcifera* containing considerable amounts of PCs and phosphatidylethanolamine (PE) induce the production of benzyl benzoate in rice, which acts as an ovicidal substance (Yang et al., 2014). Application of rhamnolipids, which are glycolipids produced by various bacteria, induces a SAR against *P. syringae*, *B. cinerea* and the oomycete *Hyaloperonospora arabidopsidis* in *Arabidopsis* (Sanchez et al., 2012). These examples illustrate the ability of chemically different lipids to elicit various immune responses in plants and demonstrate a potentially conserved

mechanism upon recognition of lipidic PAMPs and EAMPs that leads to SAR establishment against microbial pathogens in *Arabidopsis*.

EE-induced SAR against *B. cinerea* is dependent on LecRK-I.8 and this effect was already reported for EE-induced SAR against *Pst*, in accordance with a diminished egg-induced SA accumulation in *lecrk-I.8* mutant (Gouhier-Darimont et al., 2019). In addition to SA accumulation, several immune responses including ROS production, cell death activation and defence gene expression are reduced in *lecrk-I.8* following EE and PC treatments (Gouhier-Darimont et al., 2019; Stahl et al., 2020), showing that LecRK-I.8 is a crucial component for the perception of eggs. Another study described LecRK-I.8 as a potential sensor for extracellular NAD^+ ($eNAD^+$) in *Arabidopsis* (Wang et al., 2017). Intriguingly, the authors show that bacterial-induced expression of *PR1* is reduced but that SAR induction is not affected in *lecrk-I.8* mutants (Wang et al., 2017). However, when treated with flg22, *lecrk-I.8* mutant showed normal defence gene expression (Gouhier-Darimont et al., 2019), illustrating possible divergent roles of LecRK-I.8 depending on the biotic stress encountered. Interestingly, exogenously applied NAD^+ can move systemically and induce a SAR through the action of a LecRK from another clade, LecRK-VI.2 (Wang et al., 2019).

MPK3 and MPK6 are involved in PTI and ETI during pathogen infection and function downstream of receptors by transmitting extracellular stimuli into intracellular responses (Tsuda and Katagiri, 2010; Meng and Zhang, 2013). We show here that EE-induced SAR is abolished in *mpk3-1* and *mpk6-2* mutants. These two kinases were already shown to be important for the priming of defence responses and SAR induction against bacterial pathogens (Menke et al., 2004; Beckers et al., 2009). MPK3/MPK6 activation in local leaves phosphorylates the transcription factor WRKY33, which induces Pip and NHP production by directly binding to the promoter of *ALD1* and therefore activates SAR (Wang et al., 2018). However, this regulatory loop is induced in local leaves following infection by avirulent pathogens which trigger a sustained activation of MPK3/MPK6 and can therefore bypass SA signalling (Tsuda et al., 2013). In contrast, upon infection by virulent pathogens, which do not trigger sustained MPK3/MPK6 activation, SAR requires a functional SA signalling pathway (Wang et al., 2018). Following EE treatment, a rapid (30 min after application) phosphorylation of MPK3/MPK6 was detectable (C. Gouhier-Darimont, unpublished) and this effect was still observable 6 days after EE treatment (O. Hilfiker PhD thesis, unpublished). Moreover, MPK3/MPK6 phosphorylation was considerably reduced in the *lecrk-I.8* mutant (C. Gouhier-Darimont, unpublished). Thus, the absence of EE-induced SAR against *B. cinerea* in *mpk3-1*

and *mpk6-2* mutants could be due to compromised early signalling steps of egg perception or due to direct SAR-regulating functions of these kinases.

The activation of the SA pathway is required to establish EE-induced SAR against *B. cinerea*. This finding is somewhat surprising given that plant resistance to *B. cinerea* is generally known to require JA/ET pathways (Pieterse et al., 2012). However, although basal resistance to *B. cinerea* is not affected in the SA-deficient mutant *ics1*, some reports describe a contribution of SA signalling in defence against *B. cinerea*. In addition, treatment with the functional SA analog benzo-(1,2,3)-thiadiazole-7-carbothioic acid S-methyl ester (BTH) reduced *B. cinerea* infection (Zimmerli et al., 2001) and exogenous application of SA decreased *B. cinerea* lesion size and the *ein2-1 npr1* double mutant was more susceptible than the *ein2-1* single mutant (Ferrari et al., 2003). The transgenic line *nahG*, which degrades SA into catechol (Gaffney et al., 1993), displays a higher susceptibility to *B. cinerea*. Previous reports indicated that the expression of *nahG* in *Arabidopsis* leads to reduced ET and camalexin production, which are both important to fend off *B. cinerea* (Heck et al., 2003), thus explaining such phenotype.

Interestingly, we found that basal resistance to *B. cinerea* is reduced in the fully insensitive SA mutant *npr1-1 npr4-4D* (Fig. 3C-D), indicating that the SA pathway contributes to resistance. However, we observe a residual, yet significant EE-induced SAR in *npr1-1* mutant. In line with this finding, expression of NHP biosynthetic genes and SAR regulatory genes is not fully abolished in *npr1-1* mutant following *Psm* infection (Liu et al., 2020), suggesting that mild immune responses can still occur. On the contrary, EE-induced SAR was abolished in mutants impaired in local induction of SA following EE treatment (*ics1, ics1 ics2, nahG*) and in *npr1-1 npr4-4D*, which is completely blocked in *Psm*-induced SAR as well as in NHP and SAR gene expression (Liu et al., 2020). These results thus highlight the importance of SA for systemic immune responses against *B. cinerea* and *Pst*.

Arabidopsis accumulates high SA levels in response to *Pst* infection (Vlot et al., 2009). Consistent with the SA requirement for SAR establishment, we found that inoculation of *Arabidopsis* local leaves with *Pst* is sufficient to induce systemic protection against *B. cinerea*. Whether a first inoculation with other SA-inducing pathogens can induce a similar SAR against *B. cinerea* would be interesting to test. However, a first infection with *B. cinerea* does not induce SAR against subsequent infection with *Pst* or *B. cinerea* (Govrin and Levine, 2002), showing the importance of local SA induction to establish SAR.

Our data support a role for the SA pathway in controlling *B. cinerea* infection. Since SA is known to control biotrophic pathogens, we postulate that it may target an early

biotrophic phase of this fungus. Indeed, phenotypic and transcriptomic analyses of *Arabidopsis* plants infected with *B. cinerea* isolates support a more intricate role of JA and SA pathways in resistance (Zhang et al., 2017). There is also growing evidence that the trophic lifestyle of *B. cinerea* is more plastic than previously thought (van Kan et al., 2014; Veloso and van Kan, 2018) and this may explain why the SA pathway contributes to defence against this fungus. Since we found that *ein2-1* and *aos* mutants are defective in EE-induced SAR but displayed a higher susceptibility to *B. cinerea*, as previously shown (Thomma et al., 1999), we postulate that, unlike SA, JA and ET pathways are not implicated in EE-induced SAR signalling but are rather important for basal and local resistance to this pathogen, after the initial biotrophic phase of infection.

Also, we demonstrate that EE-induced SAR against *B. cinerea* requires the NHP pathway, consistent with previous findings involving Pip in EE-induced SAR against *Pst* (Hilfiker et al., 2014). Pip was initially considered as being the key SAR signal (Návarová et al., 2012) but recent work has shown that NHP, is the actual SAR regulator (Chen et al., 2018; Hartmann et al., 2018). Indeed, exogenous application of Pip is sufficient to restore SAR against *Psm* in the Pip-deficient *ald1* mutant, but not in *fmo1* (Návarová et al., 2012). We also show that Pip complementation to *ald1* is not sufficient to restore SAR against *B. cinerea*, but requires the EE pretreatment, implying an additional EE-derived signal. Infiltration of SA in replacement of EE did not reproduce SAR against *B. cinerea* in any of the genotypes tested (Supplemental Fig. 5C). Metabolite infiltration in leaves might artificially fill the apoplast with active compounds and potentially affect natural signalling in such long-distance defence responses. However, local expression of the SA-marker gene *PR1* was induced, at least 4 h following SA infiltration, showing that plants are responsive to such treatments (Supplemental Fig. 5B). Consistently, previous SA infiltration and subsequent infection in the same leaves inhibit growth of *Psm* (Gruner et al., 2018). In addition, EE is applied once but remains for 5 days on the leaves, during which PCs might be released and continuously induce SA-dependent responses compared to infiltration where SA remains in the apoplast for 4 h before infection. To mimic EE-induced SA accumulation more closely it would be interesting to infiltrate leaves every day during 4 days, in accordance with the kinetics of SA accumulation following EE treatment (Bruessow et al., 2010). Another signal involved in EE-induced SAR could be ET, whose biosynthetic genes are induced following *P. brassicae* oviposition (Little et al., 2007). In addition, cultured *Arabidopsis* cells emitted ET following application of EE (F. Bruessow, unpublished). To decipher a potential involvement of ET as EE-derived signal for EE-induced SAR establishment along with Pip, the use of an *ein2-1 ald1* double mutant would be useful

and interesting to test. Contrastingly, exogenous application of NHP can restore SAR against *Psm* in both *ald1* and *fmo1* mutants, showing that NHP functions as the critical SAR regulator (Chen et al., 2018; Hartmann et al., 2018). Recently, it was shown that exogenous application of NHP triggers the upregulation of SAR-related genes in an NPR1-dependent manner, and primes plants for an enhanced defence metabolism activation (Yildiz et al., 2021). In line with this, it would be interesting to test whether exogenous application of NHP is able to restore EE-induced SAR against *B. cinerea* in *fmo1*.

In conclusion, we show that *P. brassicae* eggs induce a SAR against *B. cinerea*, using common signalling mechanisms with egg-induced SAR against *Pst* (Hilfiker et al., 2014), involving both SA and NHP pathways.

MATERIALS AND METHODS

Plant and Insect Growth Conditions

Arabidopsis thaliana (Col-0) plants were sown in moist potting compost. After seed stratification for 2 days at 4°C, plants were grown for 4 weeks in growth chambers in short day conditions (10 h light/14 h dark), under 100 µmol m^{-2} s^{-1} of light, at 20-22°C and 65% relative humidity.

Lines used in this study: *ald1* (Návarová et al., 2012), *aos* (Park et al., 2002), *ein2-1* (Guzman and Ecker, 1990), *fmo1* (Mishina and Zeier, 2006), *ics1* (*sid2-1* allele) (Nawrath and Métraux, 1999), *ics2* (Garcion et al., 2008), *lecrk-I.8* (Gouhier-Darimont et al., 2013), *mpk3-1* (Wang et al., 2007), *mpk6-2* (Liu and Zhang, 2004), *nahG* (Nawrath and Métraux, 1999), *npr1-1* (Cao et al., 1997), *npr1-1 npr4-4D* (Liu et al., 2020). All genotypes were in the Columbia (Col-0) background. The *ics1*$^{-/-}$ *ics2*$^{+/-}$ double mutant was obtained by crossing *ics1* and *ics2*, and was genotyped using a CAPS marker for *ics1* (Heck et al., 2003) and flanking primers for *ics2* T-DNA knockout (Garcion et al., 2008).

A population of the Large White butterfly *Pieris brassicae* was maintained on *Brassica oleracea* var. *gemmifera* in a greenhouse at 24°C and 65% relative humidity (Reymond et al., 2000). *Spodoptera littoralis* eggs were obtained from Syngenta (Stein AG, Switzerland).

Oviposition and Treatment with EE and PCs

For experiments with natural oviposition, 10-15 pots each containing two plants were placed in a 60 x 60 x 60 cm tent containing around 30 *P. brassicae* butterflies. After 24 h, eight plants containing one egg batch on each of two leaves were placed in a growth chamber for 4 days. Just before hatching, eggs were gently removed with a forceps and two distal leaves were infected with *B. cinerea*. Control plants were kept in the same conditions without butterflies.

For EE preparation, *P. brassicae* or *S. littoralis* eggs were crushed with a pestle in Eppendorf tubes. After centrifugation (14,000 *g* for 3 min), the supernatant (EE) was collected and stored at -20°C. For application, 2 x 2 µl of EE were spotted under the surface of each of two leaves on 4-6 plants. Plants were treated 5 days before *B. cinerea* infection. Untreated plants were used as controls.

For PC application, a PC-mix (purified from chicken egg, 840051, Avanti Polar Lipids, Alabaster, Alabama, USA) was solved in 1% DMSO, 0.5% Glycerol and 0.1% Tween 20 by sonication. 2 x 2 µl of PC (5 µg/µl), which constitutes the natural PC concentration in *P.*

brassicae EE (Stahl et al., 2020), were spotted under the surface of each of two leaves on 4-6 plants. Control plants were treated with 1% DMSO, 0.5% Glycerol and 0.1% Tween 20.

Culture of *B. cinerea,* Infection and Growth Assessment

B. cinerea strain BMM, isolated from *Pelargonium zonale* (Zimmerli et al., 2001), was grown on 1X PDA (Potato Dextrose Agar, 39 g l^{-1}, Difco) for 10-14 days in darkness at 23°C. Spores were harvested in water and filtered through wool placed in a 10 ml tip to remove hyphae. Spores were diluted in half-strength PDB (Potato Dextrose Broth, 12 g l^{-1}, Difco) to a concentration of 5 x 10^5 spores ml^{-1} for inoculation. One 5 µl droplet of spore suspension was deposited on the adaxial surface of two leaves per plant. Inoculated plants were kept under a water-sprayed transparent lid to maintain high humidity in a growth chamber under dim light (around 2 µmol m^{-2} s^{-1}) during the whole time of infection. Lesion size measurements were made using ImageJ software version 2.0.0-rc-65/1.51u (http://imagej.nih.gov/ij).

To visualize *B. cinerea* structures, inoculated leaves were stained with lactophenol-trypan blue during 2 h at 37°C. Stained leaves were cleared in boiling 95% EtOH and stored in 70% EtOH. Observation of *B. cinerea* hyphae was done using a Leica MZ16A stereomicroscope fitted with a DFC310FX camera (Leica Microsystems). Images were then analyzed with ImageJ.

To quantify *B. cinerea* growth, total RNA was extracted using a ReliaPrep™ RNA Tissue Miniprep System (Promega). For cDNA synthesis, 500 ng of total RNA was reverse-transcribed using M-MLV reverse transcriptase (Invitrogen) in a final volume of 15.25 µl. Each cDNA sample was generated in triplicate and diluted eightfold with water. Quantitative real-time PCR analysis was performed in a final volume of 20 µl containing 2 µl of cDNA, 0.2 µM of each primer, 0.03 µM of reference dye and 10 µl of Brilliant III Ultra Fast SYBR Green qPCR Master Mix (Agilent). Reactions were performed using an Mx3000P real-time PCR machine (Agilent) with the following program: 95°C for 3 min, then 40 cycles of 10 sec at 95°C and 20 sec at 60°C. Relative mRNA abundance of *B.c.* Tubulin was normalized to the housekeeping gene *PUX1* (At3g27310) as described in Windram et al., 2012. The following primers were used: *B.c. Tub* (Broad MIT ID: BC1G_00122) forward: 5'-TTCCATGAAGGAGGTTGAGG-3', reverse: 5'-TACCAACGAAGGTGGAGGAC-3'; PUX1 (At3g27310) forward: 5'-AATGTTGCCTCCAATGTGTGA-3', reverse: 5'-TTTTTACCGCCTTTTGGCTAC-3'.

Infiltration with *Pseudomonas syringae*

Pseudomonas syringae pv. *tomato* DC3000 was streaked from a glycerol stock onto plate containing Luria Bertani (LB) medium with rifampicin 50 µg/ml. One day before infection, single colony from LB plate was inoculated into 5 ml of liquid LB medium with rifampicin 1µl/ml and placed at 200 rpm, at 28°C over night (O/N). Two ml of O/N culture were transferred into a 2 ml Eppendorf tube and centrifuged at 7,000 rpm for 3 min. The pellet was re-suspended in 2 ml of 10 mM $MgCl_2$. Bacterial concentration was adjusted to an OD600 of 0.0005. Two days before *B. cinerea* infection, two leaves per plant were infiltrated using a 1 ml needleless syringe until the leaves are wet and translucid.

Exogenous Application of Pip

One day prior to *B. cinerea* infection, 10 ml of a 1 mM D,L-Pip (Sigma-Aldrich) solution was pipetted onto each pot containing one plant. Control plants were supplemented with 10 ml of water.

SA Quantification and Infiltration

The biosensor bacteria *Acinetobacter* sp. ADPWH_*lux* (Huang et al., 2005) was used to quantify SA (DeFraia et al., 2008). Briefly, 6 leaf discs from 3 plants (2 leaf discs per plant) (0.7 cm, ca. 20 mg) were frozen, ground in liquid nitrogen and extracted in 0.1 M sodium acetate buffer (pH 5.6). Extracts were then centrifuged at 4 °C for 15 min at 16,000 *g*. About 50 µl of extracts were incubated with 5 µl of β-Glucosidase from almonds (0.5 U/µl in acetate buffer, Sigma- Aldrich) for 90 min at 37 °C to release SA from SA glucoside. Twenty ml of extract was then mixed with 60 µl of LB and 50 µl of a culture of *Acinetobacter* sp. ADPWH_*lux* (OD600=0.4), and incubated at 37°C for 60 min. Finally, luminescence was measured using a 485 ± 10 nm filter for 1 s. A SA standard curve diluted in untreated *ics1* extracts ranging from 0 to 60 ng was read in parallel to allow quantification. SA amounts in samples were estimated by fitting a third-order polynomial regression on the standards.

For SA infiltration, Col-0, *ald1*, *fmo1, ics1 ics2* or the GUS reporter line PR1::GUS (Bruessow et al., 2010) were infiltrated with 0.25 or 0.5 mM solutions in the abaxial side of two leaves per plant with a 1 ml needleless syringe. H_2O was infiltrated as control. After 4 h,

plants were harvested for GUS analysis (Bruessow et al., 2010) and SA quantification, or further infected with *B. cinerea*.

Statistical analyses

Data were analysed using R software version 3.5.2 (http://www.R-project.org). Normal distribution and variance homogeneity of data were evaluated with Shapiro-Wilk and Levene's test, respectively. If not normal, data were log-transformed to ensure analyses with parametric tests.

To compare CTL vs EE within the same genotype in SAR bioassays, we used a linear mixed model fit by the restricted maximum likelihood (REML) algorithm (package "lme4" in R) using plant treatment as a fixed factor and experimental block as a random factor.

AUTHOR CONTRIBUTIONS

Esteban Alfonso performed the experiments and analysed data for all the figures except Figure 3E and Supplemental Figure 5A that were performed by Etienne Bellani and Supplemental Figure 5B that was performed by Elia Stahl.
Esteban Alfonso wrote the Chapter and Philippe Reymond reviewed and edited the text.

REFERENCES

Bandoly M, Hilker M, Steppuhn A (2015) Oviposition by *Spodoptera exigua* on *Nicotiana attenuata* primes induced plant defence against larval herbivory. Plant J **83**: 661–672

Beckers GJM, Jaskiewicz M, Liu Y, Underwood WR, He SY, Zhang S, Conrath U (2009) Mitogen-Activated Protein Kinases 3 and 6 Are Required for Full Priming of Stress Responses in *Arabidopsis thaliana*. The Plant Cell **21**: 944–953

Bonnet C, Lassueur S, Ponzio C, Gols R, Dicke M, Reymond P (2017) Combined biotic stresses trigger similar transcriptomic responses but contrasting resistance against a chewing herbivore in Brassica nigra. BMC Plant Biol **17**: 127

Bruessow F, Gouhier-Darimont C, Buchala A, Metraux J-P, Reymond P (2010) Insect eggs suppress plant defence against chewing herbivores: Insect eggs and plant defence. The Plant Journal **62**: 876–885

Bürger M, Chory J (2019) Stressed Out About Hormones: How Plants Orchestrate Immunity. Cell Host & Microbe **26**: 163–172

Cao H, Glazebrook J, Clarke JD, Volko S, Dong X (1997) The Arabidopsis NPR1 Gene That Controls Systemic Acquired Resistance Encodes a Novel Protein Containing Ankyrin Repeats. Cell **88**: 57–63

Chen Y-C, Holmes EC, Rajniak J, Kim J-G, Tang S, Fischer CR, Mudgett MB, Sattely ES (2018) *N* -hydroxy-pipecolic acid is a mobile metabolite that induces systemic disease resistance in *Arabidopsis*. Proc Natl Acad Sci USA **115**: E4920–E4929

Chrispeels MJ, Raikhelb NV (1991) Lectins, Lectin Genes, and Their Role in Plant Defense. 9

DeFraia CT, Schmelz EA, Mou Z (2008) A rapid biosensor-based method for quantification of free and glucose-conjugated salicylic acid. Plant Methods **4**: 28

Ding P, Rekhter D, Ding Y, Feussner K, Busta L, Haroth S, Xu S, Li X, Jetter R, Feussner I, et al (2016) Characterization of a Pipecolic Acid Biosynthesis Pathway Required for Systemic Acquired Resistance. Plant Cell **28**: 2603–2615

Ferrari S, Plotnikova JM, De Lorenzo G, Ausubel FM (2003) *Arabidopsis* local resistance to *Botrytis cinerea* involves salicylic acid and camalexin and requires *EDS4* and *PAD2* , but not *SID2* , *EDS5* or *PAD4*: *Local* Arabidopsis *response to* Botrytis. The Plant Journal **35**: 193–205

Fu ZQ, Dong X (2013) Systemic Acquired Resistance: Turning Local Infection into Global Defense. Annu Rev Plant Biol **64**: 839–863

Gaffney T, Friedrich L, Vernooij B, Negrotto D, Nye G, Uknes S, Ward E, Kessmann H, Ryals J (1993) Requirement of Salicylic Acid for the Induction of Systemic Acquired Resistance. Science **261**: 754–756

Garcion C, Lohmann A, Lamodière E, Catinot J, Buchala A, Doermann P, Métraux J-P (2008) Characterization and Biological Function of the *ISOCHORISMATE SYNTHASE2* Gene of Arabidopsis. Plant Physiol **147**: 1279–1287

Gouhier-Darimont C, Schmiesing A, Bonnet C, Lassueur S, Reymond P (2013) Signalling of Arabidopsis thaliana response to Pieris brassicae eggs shares similarities with PAMP-triggered immunity. Journal of Experimental Botany **64**: 665–674

Gouhier-Darimont C, Stahl E, Glauser G, Reymond P (2019) The Arabidopsis Lectin Receptor Kinase LecRK-I.8 Is Involved in Insect Egg Perception. Front Plant Sci **10**: 623

Govrin EM, Levine A (2002) Infection of Arabidopsis with a necrotrophic pathogen, Botrytis cinerea, elicits various defense responses but does not induce systemic acquired resistance (SAR). 10

Gruner K, Zeier T, Aretz C, Zeier J (2018) A critical role for Arabidopsis MILDEW RESISTANCE LOCUS O2 in systemic acquired resistance. Plant J **94**: 1064–1082

Guzman P, Ecker JR (1990) Exploiting the Triple Response of Arabidopsis to Identify Ethylene-Related Mutants. 12

Hartmann M, Kim D, Bernsdorff F, Ajami-Rashidi Z, Scholten N, Schreiber S, Zeier T, Schuck S, Reichel-Deland V, Zeier J (2017) Biochemical Principles and Functional Aspects of Pipecolic Acid Biosynthesis in Plant Immunity. Plant Physiol **174**: 124–153

Hartmann M, Zeier J (2019) N-hydroxypipecolic acid and salicylic acid: a metabolic duo for systemic acquired resistance. Current Opinion in Plant Biology **50**: 44–57

Hartmann M, Zeier T, Bernsdorff F, Reichel-Deland V, Kim D, Hohmann M, Scholten N, Schuck S, Bräutigam A, Hölzel T, et al (2018) Flavin Monooxygenase-Generated N-Hydroxypipecolic Acid Is a Critical Element of Plant Systemic Immunity. Cell **173**: 456-469.e16

Heck S, Grau T, Buchala A, Metraux J-P, Nawrath C (2003) Genetic evidence that expression of NahG modifies defence pathways independent of salicylic acid biosynthesis in the Arabidopsis-Pseudomonas syringae pv. tomato interaction. Plant J **36**: 342–352

Hilfiker O, Groux R, Bruessow F, Kiefer K, Zeier J, Reymond P (2014) Insect eggs induce a systemic acquired resistance in Arabidopsis. Plant J **80**: 1085–1094

Hilker M, Fatouros NE (2016) Resisting the onset of herbivore attack: plants perceive and respond to insect eggs. Current Opinion in Plant Biology **32**: 9–16

Howe GA, Jander G (2008) Plant Immunity to Insect Herbivores. Annu Rev Plant Biol **59**: 41–66

Huang WE, Wang H, Zheng H, Huang L, Singer AC, Thompson I, Whiteley AS (2005) Chromosomally located gene fusions constructed in Acinetobacter sp. ADP1 for the detection of salicylate. Environ Microbiol **7**: 1339–1348

van Kan JAL, Shaw MW, Grant-Downton RT (2014) *Botrytis* species: relentless necrotrophic thugs or endophytes gone rogue? Molecular Plant Pathology **15**: 957–961

Little D, Gouhier-Darimont C, Bruessow F, Reymond P (2007) Oviposition by Pierid Butterflies Triggers Defense Responses in Arabidopsis. Plant Physiol **143**: 784–800

Liu Y, Sun T, Sun Y, Zhang Y, Radojičić A, Ding Y, Tian H, Huang X, Lan J, Chen S, et al (2020) Diverse Roles of the Salicylic Acid Receptors NPR1 and NPR3/NPR4 in Plant Immunity. Plant Cell **32**: 4002–4016

Liu Y, Zhang S (2004) Phosphorylation of 1-Aminocyclopropane-1-Carboxylic Acid Synthase by MPK6, a Stress-Responsive Mitogen-Activated Protein Kinase, Induces Ethylene Biosynthesis in Arabidopsis[W]. The Plant Cell **16**: 3386–3399

Lortzing V, Oberländer J, Lortzing T, Tohge T, Steppuhn A, Kunze R, Hilker M (2019) Insect egg deposition renders plant defence against hatching larvae more effective in a salicylic acid-dependent manner. 14

Meng X, Zhang S (2013) MAPK Cascades in Plant Disease Resistance Signaling. 25

Menke FLH, van Pelt JA, Pieterse CMJ, Klessig DF (2004) Silencing of the Mitogen-Activated Protein Kinase MPK6 Compromises Disease Resistance in Arabidopsis. Plant Cell **16**: 897–907

Mishina TE, Zeier J (2006) The Arabidopsis Flavin-Dependent Monooxygenase FMO1 Is an Essential Component of Biologically Induced Systemic Acquired Resistance. Plant Physiol **141**: 1666–1675

Návarová H, Bernsdorff F, Döring A-C, Zeier J (2012) Pipecolic Acid, an Endogenous Mediator of Defense Amplification and Priming, Is a Critical Regulator of Inducible Plant Immunity. Plant Cell **24**: 5123–5141

Nawrath C, Métraux J-P (1999) Salicylic Acid Induction–Deficient Mutants of Arabidopsis Express PR-2 and PR-5 and Accumulate High Levels of Camalexin after Pathogen Inoculation. 13

Park J-H, Halitschke R, Kim HB, Baldwin IT, Feldmann KA, Feyereisen R (2002) A knock-out mutation in allene oxide synthase results in male sterility and defective wound signal transduction in Arabidopsis due to a block in jasmonic acid biosynthesis. The Plant Journal 12

Pieterse CMJ, Van der Does D, Zamioudis C, Leon-Reyes A, Van Wees SCM (2012) Hormonal Modulation of Plant Immunity. Annu Rev Cell Dev Biol **28**: 489–521

Reymond P (2013) Perception, signaling and molecular basis of oviposition-mediated plant responses. Planta **238**: 247–258

Reymond P, Bodenhausen N, Van Poecke RMP, Krishnamurthy V, Dicke M, Farmer EE (2004) A Conserved Transcript Pattern in Response to a Specialist and a Generalist Herbivore. Plant Cell **16**: 3132–3147

Reymond P, Weber H, Damond M, Farmer EE (2000) Differential Gene Expression in Response to Mechanical Wounding and Insect Feeding in Arabidopsis. 14

Sanchez L, Courteaux B, Hubert J, Kauffmann S, Renault J-H, Clément C, Baillieul F, Dorey S (2012) Rhamnolipids Elicit Defense Responses and Induce Disease Resistance against Biotrophic, Hemibiotrophic, and Necrotrophic Pathogens That Require Different Signaling Pathways in Arabidopsis and Highlight a Central Role for Salicylic Acid. Plant Physiology **160**: 1630–1641

Shah J, Zeier J (2013) Long-distance communication and signal amplification in systemic acquired resistance. Front Plant Sci. doi: 10.3389/fpls.2013.00030

Stahl E, Brillatz T, Ferreira Queiroz E, Marcourt L, Schmiesing A, Hilfiker O, Riezman I, Riezman H, Wolfender J-L, Reymond P (2020) Phosphatidylcholines from Pieris brassicae eggs activate an immune response in Arabidopsis. eLife **9**: e60293

Thomma BPHJ, Eggermont K, Tierens KFM-J, Broekaert WF (1999) Requirement of Functional *Ethylene-Insensitive 2* Gene for Efficient Resistance of Arabidopsis to Infection by *Botrytis cinerea*. Plant Physiol **121**: 1093–1101

Tsuda K, Katagiri F (2010) Comparing signaling mechanisms engaged in pattern-triggered and effector-triggered immunity. Current Opinion in Plant Biology **13**: 459–465

Tsuda K, Mine A, Bethke G, Igarashi D, Botanga CJ, Tsuda Y, Glazebrook J, Sato M, Katagiri F (2013) Dual Regulation of Gene Expression Mediated by Extended MAPK Activation and Salicylic Acid Contributes to Robust Innate Immunity in Arabidopsis thaliana. PLoS Genet **9**: e1004015

Veloso J, van Kan JAL (2018) Many Shades of Grey in Botrytis–Host Plant Interactions. Trends in Plant Science **23**: 613–622

Vlot AC, Dempsey DA, Klessig DF (2009) Salicylic Acid, a Multifaceted Hormone to Combat Disease. Annu Rev Phytopathol **47**: 177–206

Vlot AC, Sales JH, Lenk M, Bauer K, Brambilla A, Sommer A, Chen Y, Wenig M, Nayem S (2020) Systemic propagation of immunity in plants. New Phytologist 17

Wang C, Huang X, Li Q, Zhang Y, Li J-L, Mou Z (2019) Extracellular pyridine nucleotides trigger plant systemic immunity through a lectin receptor kinase/BAK1 complex. Nat Commun **10**: 4810

Wang C, Zhou M, Zhang X, Yao J, Zhang Y, Mou Z (2017) A lectin receptor kinase as a potential sensor for extracellular nicotinamide adenine dinucleotide in Arabidopsis thaliana. eLife **6**: e25474

Wang H, Ngwenyama N, Liu Y, Walker JC, Zhang S (2007) Stomatal Development and Patterning Are Regulated by Environmentally Responsive Mitogen-Activated Protein Kinases in Arabidopsis W. 12

Wang Y, Schuck S, Wu J, Yang P, Döring A-C, Zeier J, Tsuda K (2018) A MPK3/6-WRKY33-ALD1-Pipecolic Acid Regulatory Loop Contributes to Systemic Acquired Resistance. Plant Cell **30**: 2480–2494

Windram O, Madhou P, McHattie S, Hill C, Hickman R, Cooke E, Jenkins DJ, Penfold CA, Baxter L, Breeze E, et al (2012) Arabidopsis Defense against Botrytis cinerea: Chronology and Regulation Deciphered by High-Resolution Temporal Transcriptomic Analysis. Plant Cell **24**: 3530–3557

Yang J-O, Nakayama N, Toda K, Tebayashi S, Kim C-S (2014) Structural determination of elicitors in *Sogatella furcifera* (Horváth) that induce Japonica rice plant varieties (*Oryza sativa* L.) to produce an ovicidal substance against *S. furcifera* eggs. Bioscience, Biotechnology, and Biochemistry **78**: 937–942

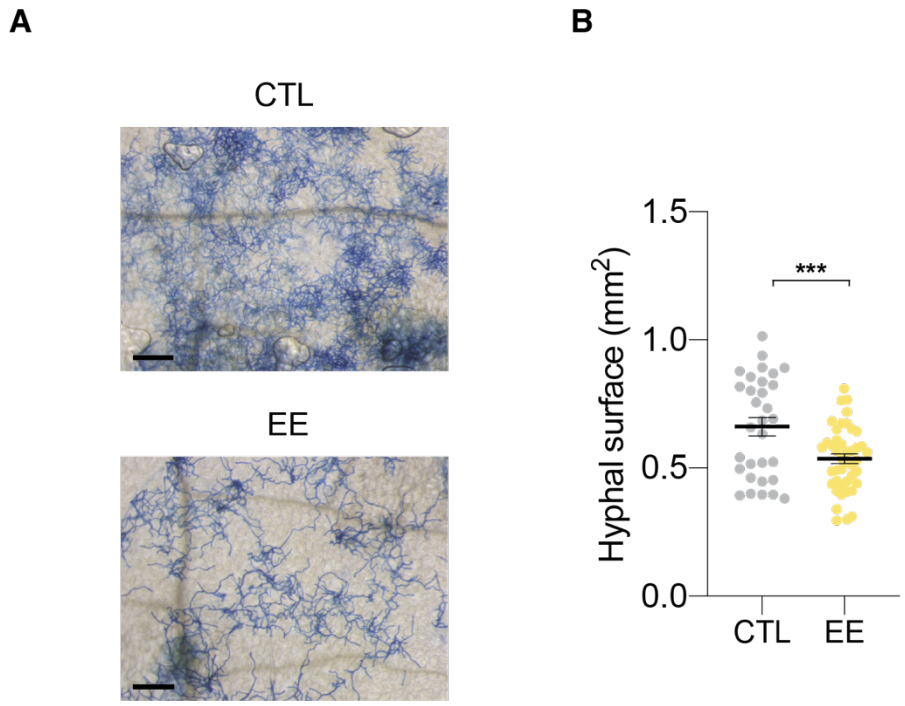
Yildiz I, Mantz M, Hartmann M, Zeier T, Kessel J, Thurow C, Gatz C, Petzsch P, Köhrer K, Zeier J (2021) The mobile SAR signal N-hydroxypipecolic acid induces NPR1-dependent transcriptional reprogramming and immune priming. Plant Physiology kiab166

Zhang W, Corwin JA, Copeland D, Feusier J, Eshbaugh R, Chen F, Atwell S, Kliebenstein DJ (2017) Plastic Transcriptomes Stabilize Immunity to Pathogen Diversity: The Jasmonic Acid and Salicylic Acid Networks within the Arabidopsis/ *Botrytis* Pathosystem. Plant Cell **29**: 2727–2752

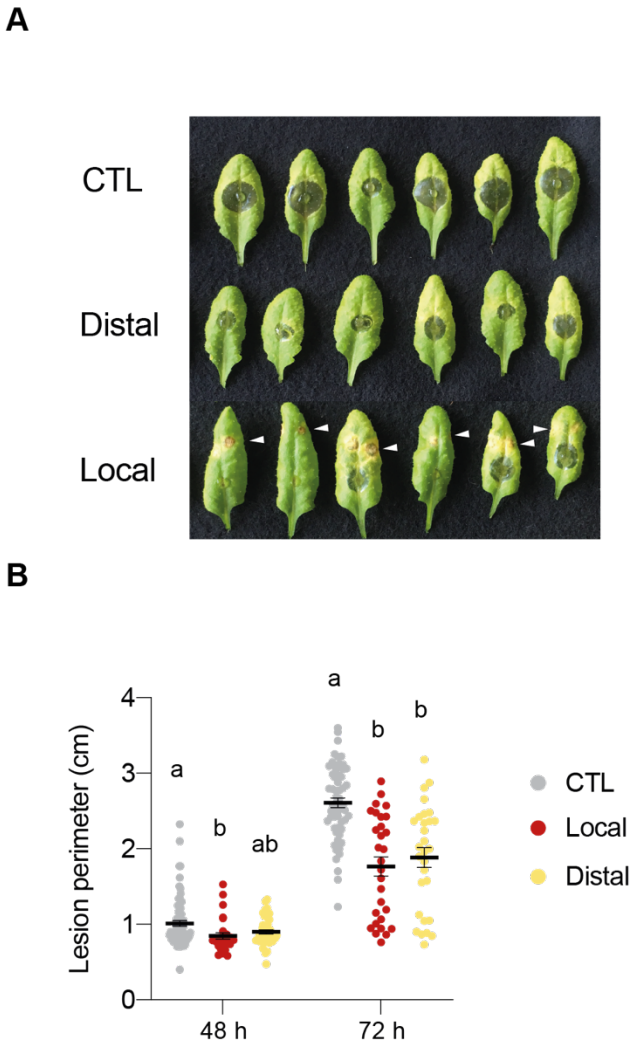
Zhou J-M, Zhang Y (2020) Plant Immunity: Danger Perception and Signaling. Cell **181**: 978–989

Zimmerli L, Métraux J-P, Mauch-Mani B (2001) β-Aminobutyric Acid-Induced Protection of Arabidopsis against the Necrotrophic Fungus Botrytis cinerea. Plant Physiology **126**: 517–523

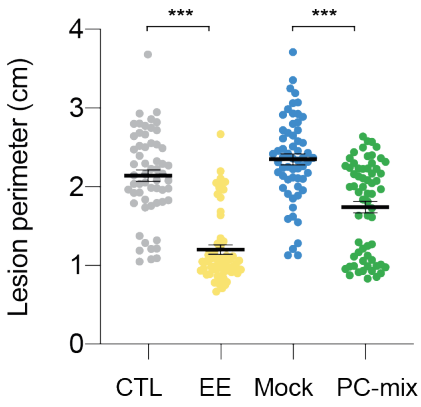
SUPPLEMENTAL DATA



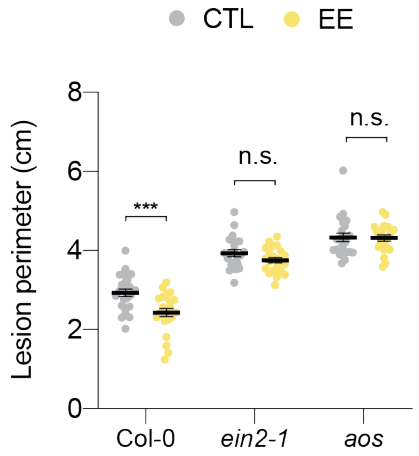
Supplemental Figure 1. *B. cinerea* hyphal growth.
(**A**) Photographs of stained hyphae on control plants (top) and plants pretreated with EE (bottom, distal leaf), 2 days post-inoculation. Scale bar: 200 µm. (**B**) Plants were pretreated with EE and hyphal growth was measured 2 days after inoculation. Hyphae were stained by trypan blue and the surface of hyphae was quantified with ImageJ. Values shown are means ± SE of three independent experiments (n = 8-14 leaves per experiments). Significant differences between control and treated plants are indicated (linear mixed model, *** P<0.001). Dots indicate individual values.



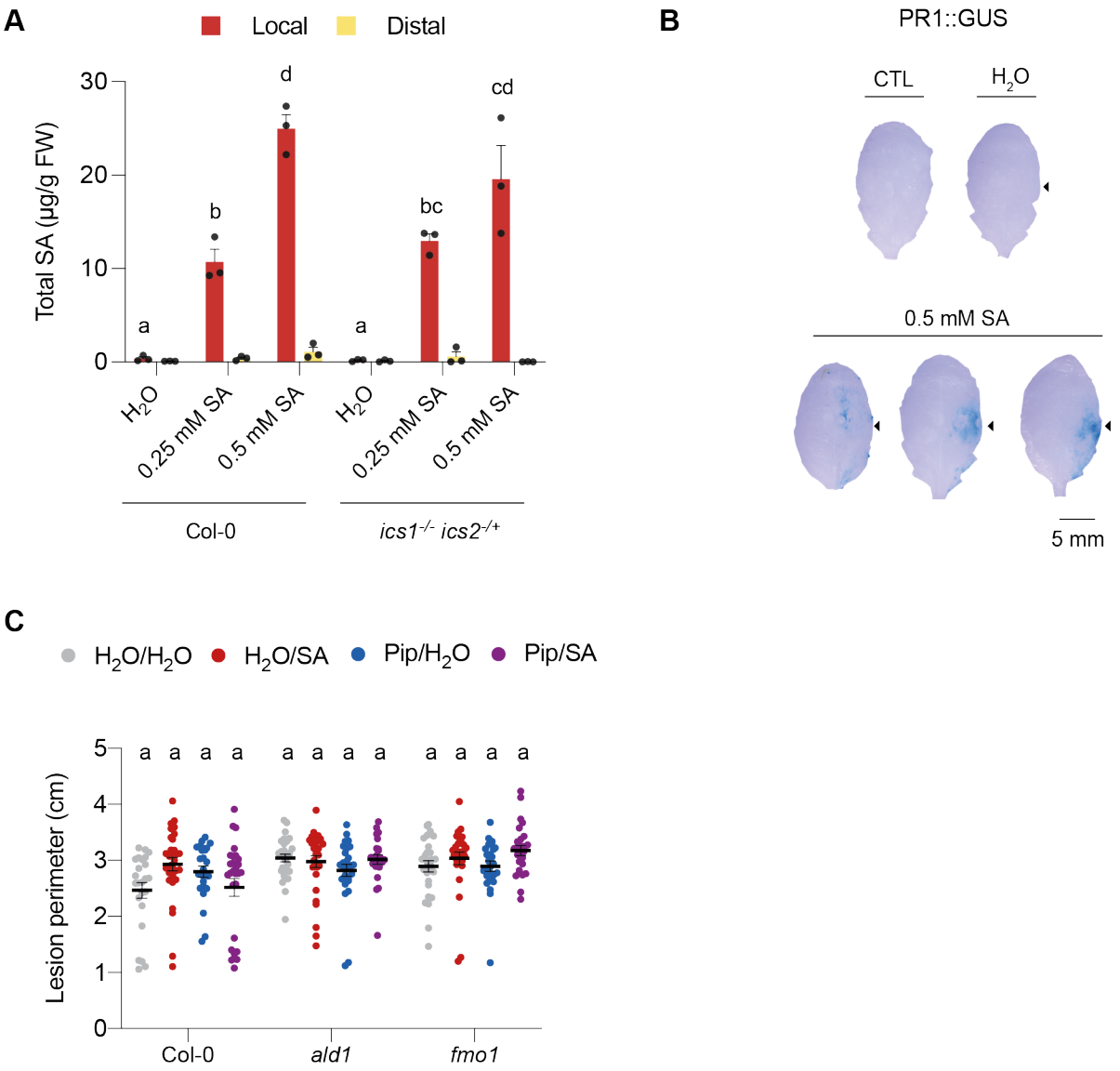
Supplemental Figure 2. Time-course of EE-induced reduction of *B. cinerea* infection.
(**A**) A solution of *B. cinerea* spores was deposited on untreated plants (CTL), on leaves distal to *P. brassicae* EE-treated leaves, or on EE-treated leaves. White arrows indicate the application site of the EE. Photographs were taken 72 h after infection. (**B**) Lesion perimeter measurement of control leaves (CTL), EE-treated leaves and leaves distal from EE-treated plants. Means ± SE of three independent experiments are shown (n = 8-37 leaves per experiment). Different letters indicate significant differences at $P<0.05$ (two-way ANOVA followed by Tukey's Honest Significant Difference test). Dots indicate individual values.



Supplemental Figure 3. PCs application reduces *B. cinerea* infection in local leaves.
Lesion perimeter was measured in local leaves of plants pretreated for 5 days with either *P. brassicae* EE or a solution of PC-mix from chicken egg. Respective controls consisted of untreated plants (CTL) or plants treated with a mock solution (Mock). Means ± SE of three independent experiments are shown (n=16-24 leaves per experiment). Significant differences are indicated (linear mixed model, *** P<0.001). Dots indicate individual values.



Supplemental Figure 4. EE-induced SAR does not depend on ET and JA pathways.
Plant genotypes were pretreated with *P. brassicae* EE for 5 days and further infected with *B. cinerea* for 3 days. Lesion perimeter was measured in control (CTL) and distal leaves from EE-treated plants (EE). Means ± SE of three independent experiments are shown (n = 6-8 leaves per experiment). Significant differences between CTL and treatment are indicated (linear mixed model, *** P<0.001; n.s., not significant). Dots indicate individual values.



Supplemental Figure 5. Exogenous SA infiltration does not trigger EE-induced SAR.
(**A**) Infiltration of H_2O and 0.5 mM SA in PR1::GUS reporter line. Black triangles indicate which half of the leaf was infiltrated. For SA infiltration, three representative images from different plants are shown. CTL, untreated. (**B**) Plant genotypes were infiltrated with H_2O, 0.25 mM and 0.5 mM of SA in the abaxial surface of two leaves per plant for 4 h before SA quantification in local (infiltrated leaves) and distal leaves. Means ± SE of three independent experiments are shown (n = 6 leaves per experiment). The double mutant *ics1 ics2* was homozygous for *ics1* ($^{-/-}$) and heterozygous for *ics2* ($^{-/+}$). Different letters indicate significant differences between treatments in local leaves at $P<0.05$ (ANOVA followed by Tukey's Honest Significant Difference test). (**C**) H_2O or 1 mM Pip solution was applied to the soil 24 h prior infection with *B. cinerea* for 3 days. H_2O or 0.25 mM SA were infiltrated in two leaves per plant 4 h prior infection. Means ± SE of three independent experiments are shown (n = 6-12 leaves per experiment). Different letters indicate significant differences at $P<0.05$ (ANOVA followed by Tukey's Honest Significant Difference test). Dots indicate individual values.

CHAPTER 2

Camalexin is required for *Pieris brassicae* egg-induced systemic acquired resistance against *Botrytis cinerea*

Esteban Alfonso[1], Elia Stahl[1], Gaétan Glauser[2] and Philippe Reymond[1]

[1]Department of Plant Molecular Biology, University of Lausanne, Switzerland
[2]Neuchâtel Platform of Analytical Chemistry, University of Neuchâtel, Switzerland

ABSTRACT

Tryptophan (Trp)-derived indolic metabolites are important for *Arabidopsis* immunity towards microbial pathogens. In *Arabidopsis*, bacteria-induced systemic acquired resistance (SAR) is accompanied by an activation of indolic metabolism. Here, we show that *Pieris brassicae* egg extract (EE)-induced SAR against the fungal necrotroph *Botrytis cinerea* requires indolic metabolism. Indeed, EE-induced SAR is abolished in *cyp79b2 cyp79b3*, a double mutant lacking all Trp-derived metabolites. More specifically, we found that EE-induced SAR is absent in *cyp71a12 cyp71a13*, *pad3-1* and *wrky33*, which are mutants that lack camalexin, a metabolite important for plant immunity against several fungal pathogens, including *B. cinerea*. On the contrary, EE-induced SAR is conserved in various indolic mutants impaired in the biosynthesis of other Trp-derived metabolites, including indole glucosinolates and 4-hydroxy-indole-3-carbonyl nitrile. However, camalexin accumulates to similar levels in response to *B. cinerea*, independently of EE pretreatment. In addition, we also show that camalexin accumulates normally in *ald1* and *ics1*, both impaired in EE-induced SAR. Both findings raise the question of how this antifungal metabolite participates in EE-induced SAR. However, we unveil here a novel aspect of egg-induced SAR with an interesting connection to indolic metabolism.

INTRODUCTION

In nature, plants are constantly challenged with numerous herbivores and microbial pathogens but complete colonisation by these invaders is usually rare, demonstrating the robustness of plant immunity, which mostly relies on secondary metabolism. Plant secondary metabolites constitute a large group of diversified molecules deployed in response to various biotic and abiotic stresses and thus essential for fitness (Piasecka et al., 2015; Erb and Kliebenstein, 2020).

In *Arabidopsis* and other cruciferous plants, tryptophan (Trp)-derived indolic metabolism constitutes an important branch of plant immunity producing defence-relevant compounds, whose biosynthesis is activated by a broad range of pathogens and herbivores (Bednarek et al., 2011; Bednarek, 2012; Kettles et al., 2013). Trp is first converted to indole-3-acetaldoxime (IAOx) by the redundant cytochrome P450 monooxygenases CYP79B2 and CYP79B3 (Hull et al., 2000; Mikkelsen et al., 2000). From IAOx, several branches diverge leading to the synthesis of indolic glucosinolates (GS), indole-3-carboxylic acids (ICAs), indole-3-carbonyl nitriles (ICNs), the indolic phytoalexin camalexin and the phytohormone indole-3-acetic acid (IAA) (Fig. 1A) (Zhao et al., 2002; Glawischnig et al., 2004; Bednarek et al., 2005; Bender and Celenza, 2009; Rajniak et al., 2015). Consequently, the *Arabidopsis cyp79b2 cyp79b3* double mutant (*cyp79b2/b3*) is completely devoid of all Trp-derived metabolites and is highly susceptible to pathogens (Sanchez-Vallet et al., 2010; Schlaeppi et al., 2010; Frerigmann et al., 2016).

GS are amino acid-derived nitrogen- and sulfur-containing thioglucosides specific to the Brassicales order (Halkier and Gershenzon, 2006). They are present constitutively and are thus classified as phytoanticipins. *Arabidopsis* accumulates two main classes of GS, methionine-derived aliphatic GS and Trp-derived indolic GS, which are chemically stable and inactive metabolites stored in vacuoles. Plants containing GS also possess specific thioglucosidases termed myrosinases, stored in different cellular compartment, which upon tissue disruption (for instance by herbivore feeding) release the glucose moiety leading to spontaneous rearrangement of the aglucone generating toxic thiocyanates, isothiocyanates or nitriles (Bones and Rossiter, 2006). In addition to their role as anti-herbivore compounds (Schlaeppi et al., 2008; Müller et al., 2010), indolic GS breakdown products display antimicrobial activity against fungal and oomycete pathogens (Bednarek et al., 2009; Sanchez-Vallet et al., 2010; Schlaeppi et al., 2010). GS biosynthesis is regulated by several transcription factors from the MYB family. MYB28 and MYB29 regulate genes involved in aliphatic GS biosynthesis while MYB34, MYB51 and MYB122 regulate biosynthesis of indolic GS

(Mitreiter and Gigolashvili, 2021). Consequently, *myb28 myb29* double mutant (*myb28/29*) and *myb34 myb51 myb122* triple mutant (*tmyb*) are devoid of aliphatic and indolic GS, respectively (Beekwilder et al., 2008; Frerigmann and Gigolashvili, 2014).

In leaves, IAOx is dehydrated by the two monooxygenases CYP71A12 and CYP71A13 to generate indole-3-acetonitrile (IAN), a key intermediate in the biosynthesis of ICAs and camalexin (Nafisi et al., 2007; Böttcher et al., 2014). Camalexin is the most abundant phytoalexin in *Arabidopsis* (Glawischnig, 2007) and has been shown to accumulate to high levels in leaves in response to infection by the bacterial pathogen *Pseudomonas syringae*, the fungal pathogens *Botrytis cinerea* and *Alternaria brassicicola* as well as in response to abiotic stresses such as UV or silver nitrate treatments (Tsuji et al., 1992; Thomma et al., 1999; Ferrari et al., 2003; Müller et al., 2015). However, camalexin accumulation is not restricted to leaves, as roots infected with the oomycete *Pythium sylvaticum* or treated with the bacterial flagellin-derived 22-amino acid elicitor flg22 also induce camalexin biosynthesis (Bednarek et al., 2005; Millet et al., 2010; Koprivova et al., 2019). CYP71A13 shares 89% identity on the amino acid level with CYP71A12 and have both been shown to convert IAOx to IAN *in vitro* (Nafisi et al., 2007; Klein et al., 2013). Upon pathogen infection and abiotic stresses, camalexin accumulation is reduced by ~80% in *cyp71a13* mutant compared to Col-0 (Nafisi et al., 2007), whereas the *cyp71a12 cyp71a13* double mutant (*cyp71a12/a13*) is completely lacking camalexin, showing that CYP71A12 is also contributing to camalexin biosynthesis in a minor way (Müller et al., 2015). During camalexin biosynthesis, IAN is conjugated with glutathione and subsequently with cysteine to generate dihydrocamalexin (Parisy et al., 2007; Geu-Flores et al., 2011). *PHYTOALEXIN DEFICIENT 3* (*PAD3*) codes for a cytochrome P450 enzyme (also known as *CYP71B15*) that catalyses the last two steps of camalexin biosynthesis from dihydrocamalexin (Zhou et al., 1999; Schuhegger et al., 2006; Böttcher et al., 2009). Accordingly, the *pad3-1* mutant is completely lacking camalexin and is highly susceptible to fungal pathogens such as *B. cinerea* (Ferrari et al., 2007; Chassot et al., 2008). Camalexin biosynthesis is regulated by the transcription factor WRKY33, which is itself activated via phosphorylation by the pathogen-responsive mitogen-activated protein kinases 3 and 6 (MPK3/MPK6) (Ren et al., 2008; Mao et al., 2011). It has been demonstrated that WRKY33 binds to the promoter of camalexin biosynthetic genes such as *CYP71A13* and *PAD3* (Qiu et al., 2008). The *wrky33* mutant is devoid of camalexin in the early phase of *B. cinerea* infection but can accumulate even more camalexin than Col-0 48 h after the infection (Birkenbihl et al., 2012).

ICA and indole-3-carbaldehyde (ICHO) are other IAN-derived metabolites that accumulate in response to pathogen attacks (Hagemeier et al., 2001; Bednarek et al., 2005;

Stahl et al., 2016). A biosynthetic route involving CYP71B6 and ARABIDOPSIS ALDEHYDE OXIDASE 1 (AAO1) has been described (Böttcher et al., 2014). However, the *Arabidopsis* double mutant *cyp71b6 aao1* still accumulates ICHO and ICA in response to abiotic stresses such as silver nitrate and UV light treatments (Müller et al., 2019), suggesting multiple origins of these metabolites. Consequently, the role of ICHO and ICA in plant immunity is difficult to assess, although a function in post-invasive immunity was recently described in response to fungal pathogens (Pastorczyk et al., 2020; Kosaka et al., 2021).

Another route from IAOx has been described, involving CYP71A12, processing IAOx to generate the intermediate indole cyanohydrin, that is then metabolised by FLAVIN-DEPENDENT OXIDOREDUCTASE 1 (FOX1) leading to ICN formation and further hydroxylated by CYP82C2 to produce 4-OH-ICN, which contributes to disease resistance in response to *P. syringae* (Rajniak et al., 2015). However, ICNs are highly unstable and hydrolyse to ICA in aqueous or methanolic solutions, therefore contributing to the pool of ICA induced during pathogen infection (Fig. 1A) (Rajniak et al., 2015).

Activation of indolic metabolism is not only restricted to the site of infection. In *P. syringae*-infected *Arabidopsis*, the accumulation of ICHO, ICA and indole-3-ylmethylamine (I3A) could also be observed in uninfected systemic tissue (Stahl et al., 2016). Moreover, the pathogen-induced biosynthesis of camalexin, I3A and ICA is primed by exogenous application of the SAR signal pipecolic acid (Pip) (Návarová et al., 2012; Stahl et al., 2016), suggesting a connection between indolic metabolism and SAR.

We showed in Chapter 1 that *Pieris brassicae* oviposition and treatment with egg extract (EE) induce a SAR against *B. cinerea*. EE-induced SAR establishment requires functional egg detection mechanisms as well as intact salicylic acid (SA) and *N*-hydroxy-Pip (NHP) signalling pathways, but connection with defence-relevant components was not discussed. *P. brassicae* oviposition and EE treatment activate several genes involved in Trp pathway and indolic metabolism (Little et al., 2007; Stahl et al., 2020). Here, we focus on the metabolic actor of EE-induced SAR by using several *Arabidopsis* mutants impacted in various branches of the indolic metabolism. We show that camalexin is required to establish EE-induced SAR and that this compound is highly toxic *in vitro* to the *B. cinerea* strain used in this study.

RESULTS

EE-induced SAR relies on camalexin accumulation

Trp-derived indolic metabolites are important for *Arabidopsis* immunity and we tested whether they are involved in EE-induced SAR against *B. cinerea*. The *cyp79b2/b3* double mutant is blocked in the conversion of Trp to IAOx, a central molecule from which several indolic metabolites derive, including indolic GS, IAN and ICN (Fig. 1A). Strikingly, EE-induced SAR was abolished in *cyp79b2/b3* (Fig. 1B), suggesting that at least one or several indolic compounds are required to establish EE-induced SAR against *B. cinerea*. Furthermore, the mutant was more susceptible to *B. cinerea* infection in absence of EE pretreatment, confirming that Trp-derived metabolites are important for basal resistance against fungal pathogens and indicate a connection of indole metabolism and SAR.

Indolic GS have been implicated in *Arabidopsis* immunity against bacterial and fungal pathogens, including *B. cinerea* (Bednarek et al., 2009; Clay et al., 2009; Xu et al., 2016). To test their possible involvement in EE-induced SAR, we quantified indolic and aliphatic GS in Col-0 plants after EE treatment and/or *B. cinerea* infection. No significant differences were observed in control and treated plants over a time-course from 12 h to 48 h after inoculation, indicating that neither EE nor *B. cinerea* induced GS accumulation (Supplemental Fig. 1). To further test the role of GS in EE-induced SAR, we used a *myb34 myb51 myb122* triple mutant (*tmyb*), which is completely devoid of indolic GS (Supplemental Fig. 2) (Frerigmann and Gigolashvili, 2014) and a *myb28/29* double mutant, lacking aliphatic GS (Beekwilder et al., 2008). EE-induced SAR was conserved in both mutants (Fig. 1C), confirming that both GS classes are not involved in this response.

4-OH-ICN is another IAOx-derived metabolite with antimicrobial activity (Rajniak et al., 2015). It is produced in several steps that start by a CYP71A12-catalysed oxidation which generates indole cyanohydrin, an unstable compound that is then processed by FOX1 to generate ICN which is then finally hydroxylated by CYP82C2 (Fig. 1A). To test the involvement of 4-OH-ICN in our response, we used mutants impaired in every biosynthetic steps. We found that *cyp71a12*, *fox1* and *cyp82c2-2* mutants were not compromised in EE-induced SAR, thus discarding 4-OH-ICN as a SAR component (Fig. 1E).

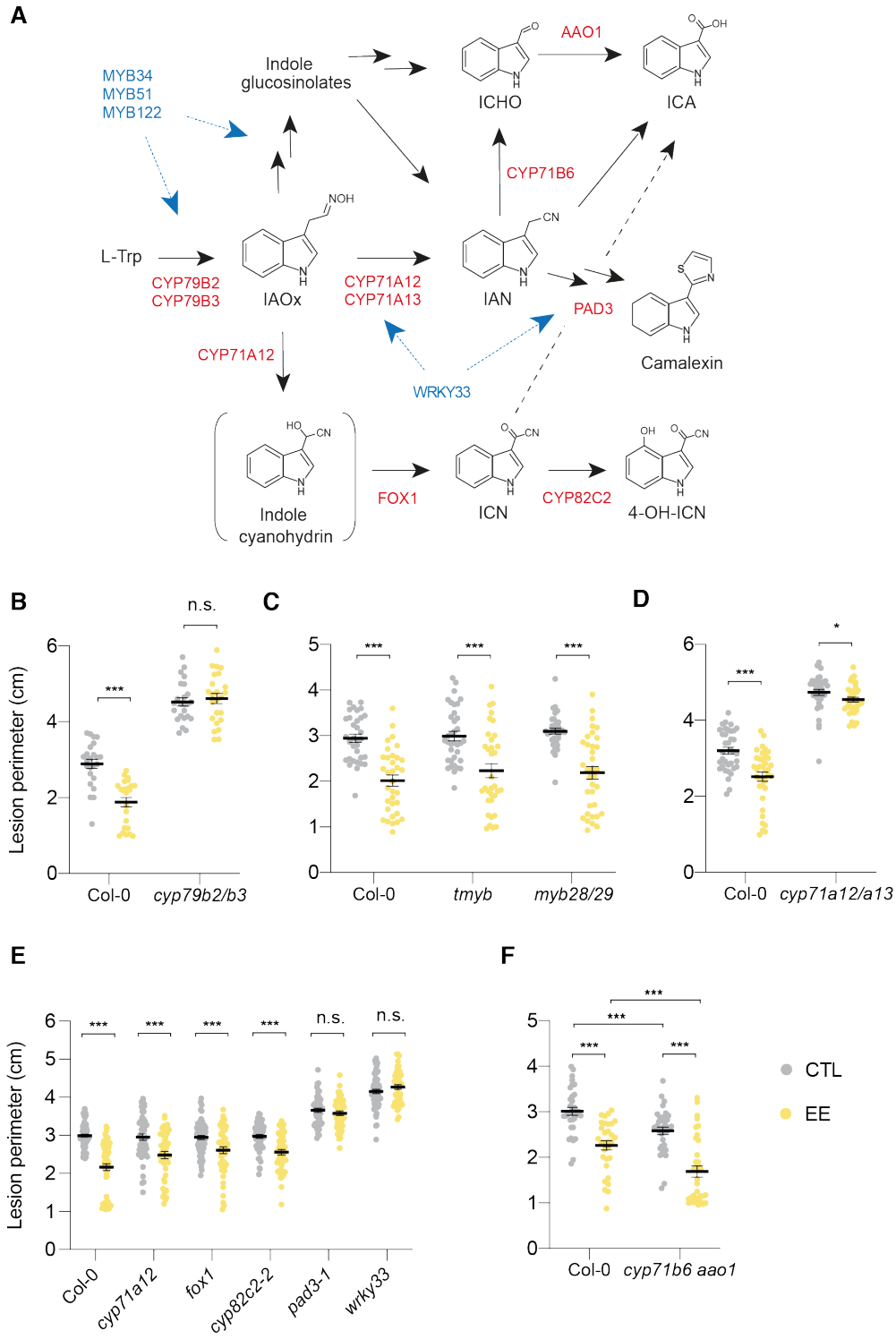


Figure 1. EE-induced SAR requires Trp-derived indolic metabolism.
(**A**) Simplified scheme of biosynthesis of tryptophan derivatives and position of biosynthesis (red) and regulatory (blue) genes tested in this study. Brackets indicate an unstable intermediate. Several arrows indicate multiple steps. L-Trp, tryptophan; IAOx, indole-3-acetaldoxime; IAN, indole-3-acetonitrile; ICN, indole carbonyl nitrile; 4-OH-ICN, 4-hydroxy-ICN; ICHO, indole-3-carbaldehyde; ICA, indole-3-carboxylic acid. (**B**-**F**) Plant genotypes were pretreated with *P. brassicae* EE for 5 days and further infected with *B. cinerea* for 3 days. Lesion perimeter was measured in control (CTL) and distal leaves from EE-treated plants (EE). Means ± SE of three independent experiments are shown (n = 8-21 leaves per experiment). Significant differences between control and treated plants are indicated (linear mixed model, *** $P<0.001$, * $P<0.05$; n.s., not significant). Dots indicate individual values. *tmyb = myb34 myb51 myb122*.

IAOx is further metabolized by CYP71A12 and CYP71A13 to generate IAN, from which camalexin, ICHO and ICA diverge (Fig. 1A). The *cyp71a12/a13* double mutant is fully deficient in camalexin production (Müller et al., 2015). EE-induced SAR was drastically reduced in *cyp71a12/a13* (Fig. 1D). This mutant was also significantly more susceptible to *B. cinerea* infection, suggesting that metabolites downstream of IAN are important for basal resistance to *B. cinerea* and to mount SAR. Although ICA and its precursor ICHO can derive from various sources, a metabolic route from IAN catalysed by CYP71B6 and AAO1 has been described (Böttcher et al., 2014). EE-induced SAR was conserved in the *cyp71b6 aao1* double mutant (Fig. 1F). Furthermore, we found that basal resistance of *cyp71b6 aao1* was increased (Fig. 1F). Finally, we tested the involvement of camalexin, which is known to inhibit *B. cinerea* growth. The *pad3-1* and *wrky33* mutants, which lack camalexin, displayed an increased susceptibility to *B. cinerea* and were fully defective in EE-induced SAR (Fig. 1E), suggesting a crucial role of camalexin for EE-induced SAR establishment.

To further confirm camalexin involvement in EE-induced SAR, we quantified camalexin in most of the indolic mutants used. We found that the *cyp71a12/a13* double mutant and *pad3-1* mutant were completely lacking camalexin (Fig. 2A-B), consistent with previous studies (Glazebrook and Ausubel, 1994; Müller et al., 2015) and correlating with the absence of EE-induced SAR in these mutants (Fig. 1D-E). On the contrary, camalexin induction following *B. cinerea* infection was comparable to Col-0 in the *tmyb* and *cyp71a12* mutants, in which EE-induced SAR was conserved (Fig. 2C). Furthermore, the *cyp71b6 aao1* double mutant accumulated significantly more camalexin in response to *B. cinerea* 48 h after infection (Fig. 2D), correlating with the increased basal resistance and the stronger EE-induced SAR observed in this double mutant (Fig. 1F). Together, these results show that EE-induced SAR establishment against *B. cinerea* requires camalexin accumulation.

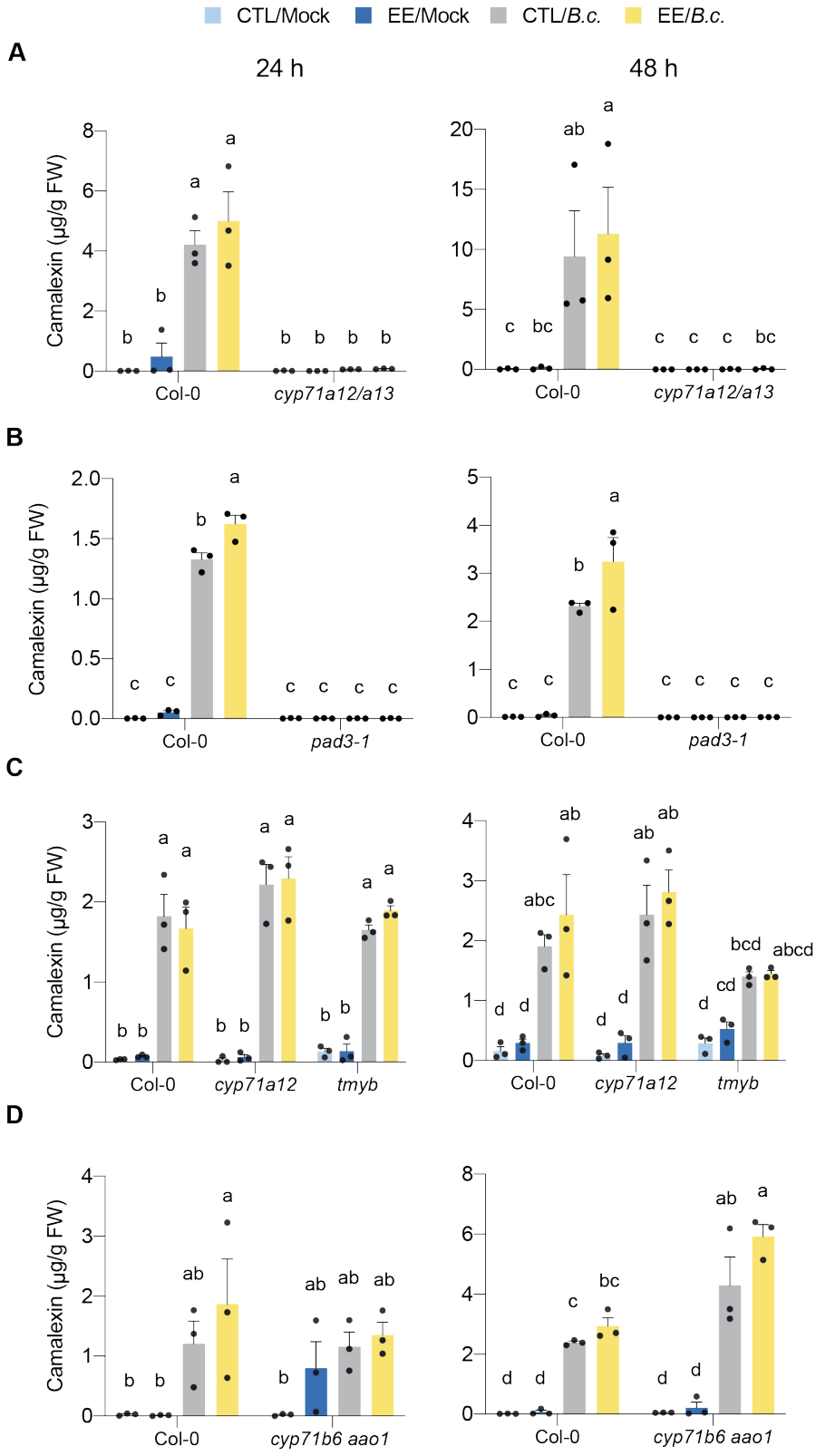


Figure 2. Camalexin accumulation in indolic mutants.
(**A-D**) Local leaves were left untreated (CTL) or pretreated with *P. brassicae* EE for 5 days (EE) and distal leaves were further inoculated with *B. cinerea* spore suspension (*B.c.*) or a mock solution (Mock) for 24 h and 48 h. Camalexin levels were measured in distal leaves. Means ± SE of three independent experiments are shown (n = 10-12 leaves per experiment). Different letters indicate significant difference at $P<0.05$ (ANOVA followed by Tukey's Honest Significant Difference test. *tmyb = myb34 myb51 myb122*.

EE pretreatment does not affect camalexin accumulation

We showed that camalexin accumulated in response to *B. cinerea* infection (Fig. 2). Interestingly, camalexin accumulated to even higher levels in EE-treated local leaves, while the induction was completely absent in distal leaves of EE-treated plants (Fig. 3A). Fungal pathogens are highly susceptible to camalexin (Pedras et al., 2011). To confirm this with the *B. cinerea* isolate used in this study, we monitored its mycelial growth in presence of purified camalexin *in vitro*. Camalexin displayed direct antifungal activity against *B. cinerea* in a dose-dependent manner (Fig. 3B). Camalexin completely inhibited *B. cinerea* growth at a concentration of 40 µg/ml, consistent with previous findings (Ferrari et al., 2003).

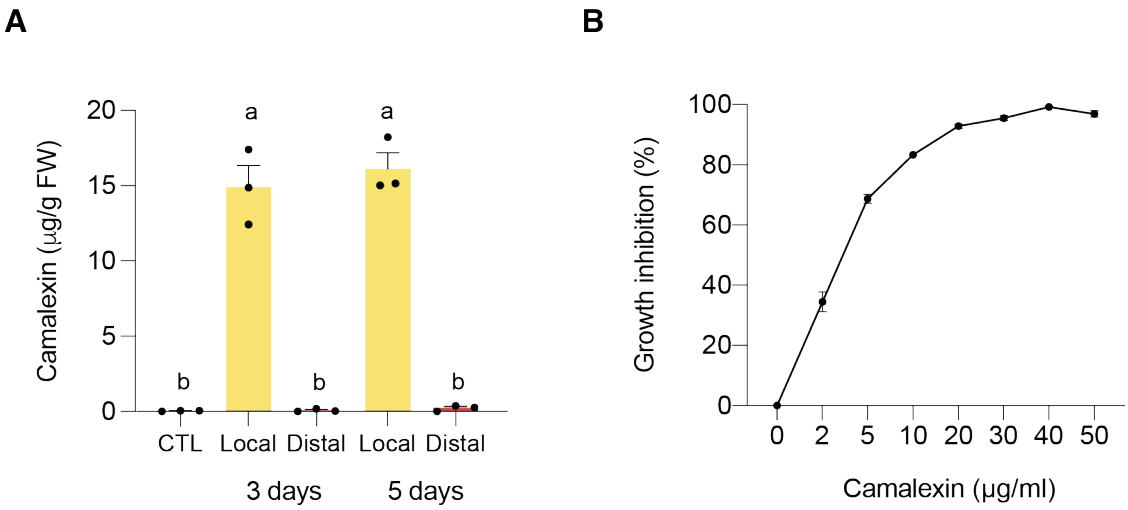


Figure 3. Camalexin accumulates in local EE-treated leaves and is toxic to *B. cinerea in vitro*.
(**A**) Col-0 plants were treated with *P. brassicae* EE for 3 days and 5 days or left untreated. Camalexin levels were measured in untreated leaves (CTL), EE-treated leaves (Local) and leaves systemic from EE-treated leaves (Distal). Means ± SE of three independent experiments are shown (n = 10-12 leaves per experiments). Different letters indicate significant difference at $P< 0.05$ (ANOVA followed by Tukey's Honest Significant Difference test). (**B**) *In vitro* growth inhibition assay. Radial growth of a *B. cinerea* colony growing on PDA plates supplemented with different concentrations of camalexin was measured after 24 h of incubation. Means ± SE of three independent experiments are shown (n = 12 measures per experiment).

B. cinerea spores start to germinate and induce the first lesion in a range time of 12 h to 18 h following leaves inoculation (Windram et al., 2012). We thus decided to measure the early *Arabidopsis* responses to *B. cinerea* 12 h post-infection. We first monitored the expression of camalexin biosynthesis genes in Col-0 and in the Pip-deficient mutant *ald1*. Expression of *PAD3* was also slightly primed by EE pretreatment in a NHP-dependent manner 12 h and 24 h post-infection (Fig. 4), although this was not corroborated by the analysis of camalexin

accumulation. *CYP71A13* expression was primed by EE pretreatment 24 h post-infection, although this result was observed in two independent experiments out of three (one experiment is shown in Fig. 4). *WRKY33*, which is a positive regulator of *PAD3* and *CYP71A13* gene expression, was not primed but rather induced systemically in response to EE 12 h following leaf infection, and this was also defective in *ald1*. In addition, we measured the expression of *atrB*, a *B. cinerea* gene coding for an ABC transporter that has been shown to export camalexin and therefore considered as a virulence factor (Stefanato et al., 2009). Interestingly, *atrB* was significantly more induced in *B. cinerea* present on Col-0 plants previously treated with EE compared to control plants, but only 24 h post-infection, in accordance with the development of *B. cinerea* during plant infection (Windram et al., 2012). This effect was not observed when *B. cinerea* was inoculated on *ald1* plants (Fig. 4).

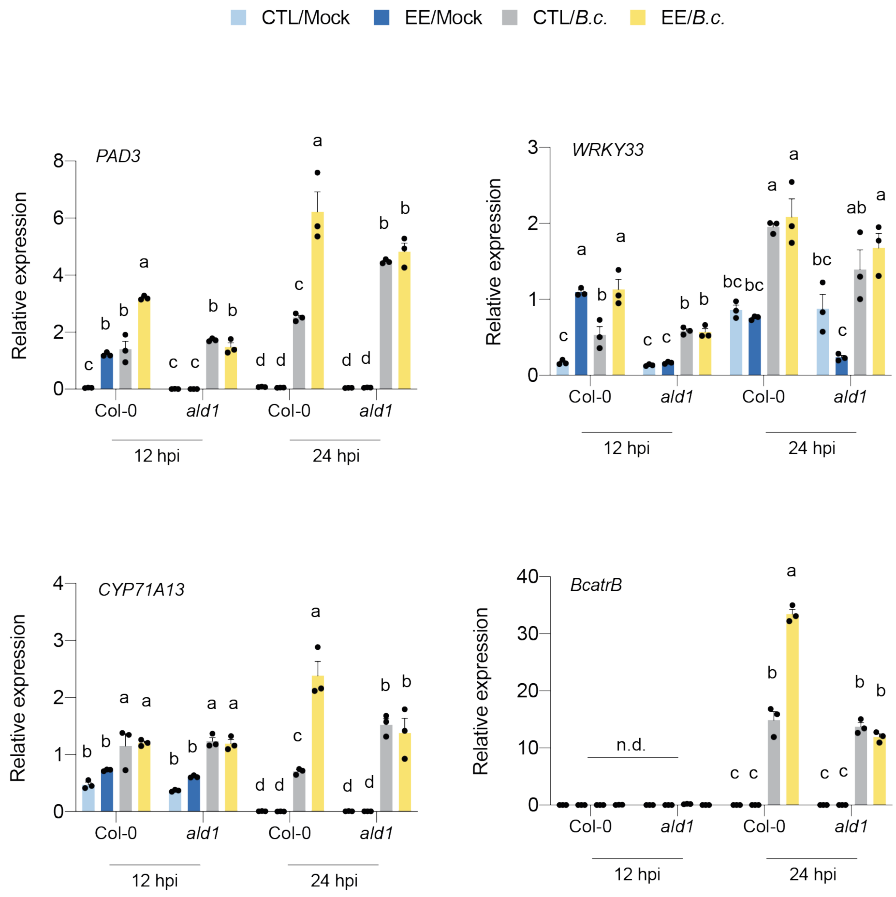


Figure 4. Expression of camalexin biosynthesis genes.
Local leaves were left untreated (CTL) or pretreated with *P. brassicae* EE for 5 days (EE) and distal leaves were further inoculated with *B. cinerea* spore suspension (*B.c.*) or a mock solution (Mock) for 12 h and 24 h post-infection (hpi). Gene expression was monitored in distal leaves. Means ± SE of three technical replicates of one experiment are shown (n = 10-12 leaves per experiment). This experiment was repeated twice with similar results. Different letters indicate significant difference at $P < 0.05$ (ANOVA followed by Tukey's Honest Significant Difference test).

We then decided to quantify camalexin in response to EE treatment and/or *B. cinerea* infection after 12 h and 24 h post-infection in Col-0 and in the SAR-deficient mutants *ald1* and *ics1*. We found a slight EE-induced priming of camalexin accumulation in Col-0 12 h post-infection (Fig. 5A). Interestingly, this increased camalexin accumulation in EE-pretreated plants was abolished in *ald1*, suggesting an involvement of the NHP pathway (Fig. 5A). However, this response was not consistent throughout the 24 h time course of *B. cinerea* infection and was not found in *ics1* mutant, where camalexin accumulated at similar levels between *B. cinerea*-infected plants previously treated with EE or not (Fig. 5A-B). Camalexin is secreted at the leaf surface where it inhibits growth of *B. cinerea* (Khare et al., 2017; He et al., 2019). To investigate whether EE pretreatment may enhance camalexin secretion, we quantified camalexin at the leaf surface of Col-0 and *ald1*, 12 and 24 h after inoculation. However, no substantial difference in camalexin secretion was observed between EE-treated and control plants, and between genotypes (Fig. 5C).

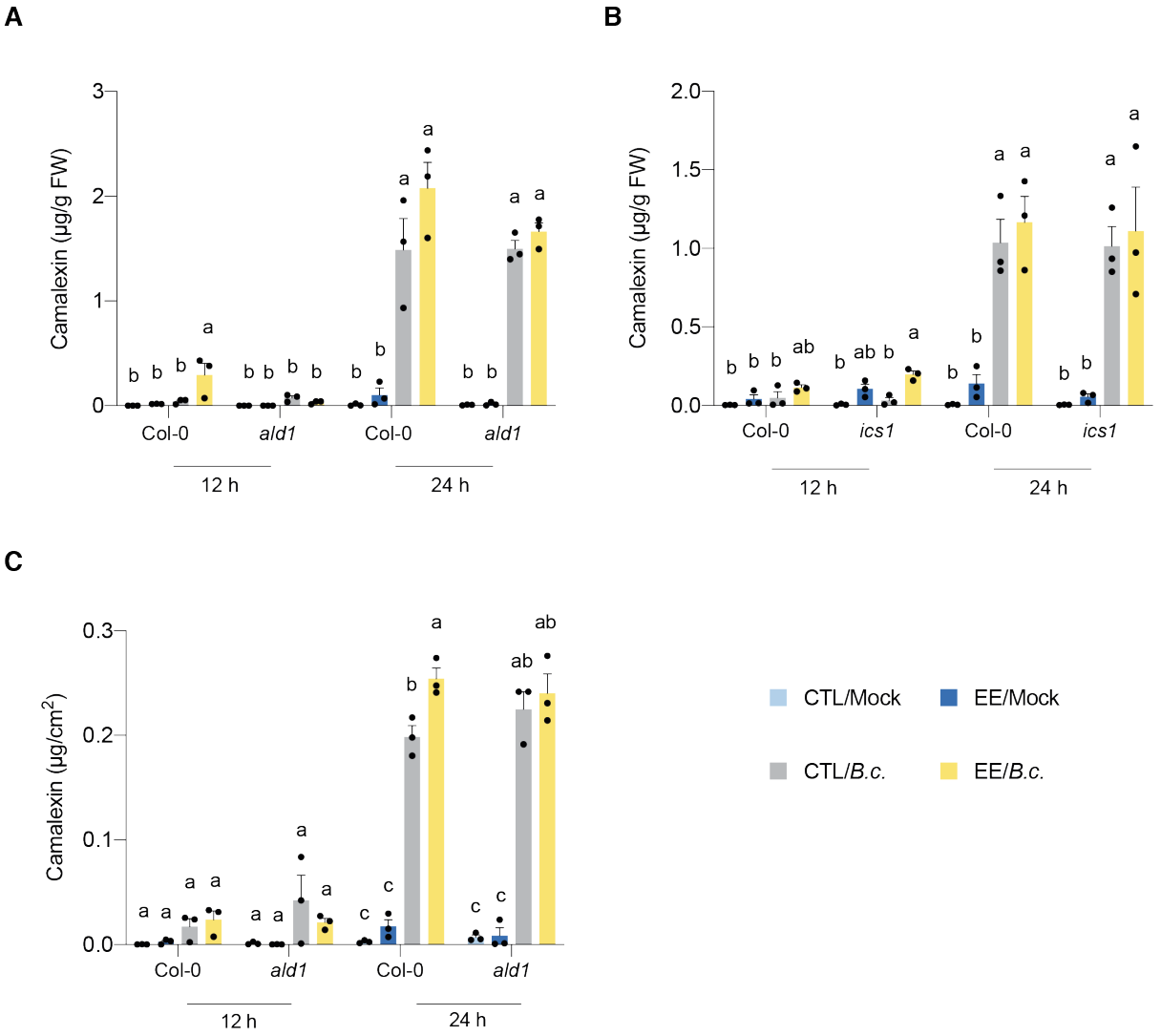


Figure 5. Camalexin accumulation after EE and *B. cinerea* treatment.
Local leaves were left untreated (CTL) or pretreated with *P. brassicae* EE for 5 days (EE) and distal leaves were further inoculated with *B. cinerea* spore suspension (*B.c.*) or a mock solution (Mock) for 12 h and 24 h. Camalexin levels were measured in distal leaves of *ald1* (**A**) and *ics1* (**B**). Means ± SE of three independent experiments are shown (n = 10-12 leaves per experiment). Different letters indicate significant difference at $P<0.05$ (ANOVA followed by Tukey's Honest Significant Difference test. (**C**) Leaf surface camalexin. Local leaves were left untreated (CTL) or pretreated with *P. brassicae* EE for 5 days (EE) and distal leaves were further inoculated with *B. cinerea* spore suspension (*B.c.*) or a mock solution (Mock) for 12 h and 24 h. Means ± SE of three independent experiments are shown (n = 8 leaves per experiment). Different letters indicate significant difference at $P< 0.05$ (ANOVA followed by Tukey's Honest Significant Difference test).

DISCUSSION

Previously, EE-induced SAR against the pathogenic bacteria *P. syringae* was associated with a stronger induction of defence gene expression, but no link with specific metabolic pathway was established (Hilfiker et al., 2014). Bacterial SAR activation was associated with a strong induction of indolic metabolism, although this was described as being a consequence and not the cause for SAR activation (Stahl et al., 2016). However, we discovered here a crucial role of camalexin, an indolic phytoalexin derived from Trp metabolism, in EE-induced SAR establishment against *B. cinerea*. Most of the indolic mutants tested in this study display a functional EE-induced SAR, in the exception of camalexin-deficient mutants, such as *cyp79b2/b3*, *cyp71a12/a13*, *pad3-1* and *wrky33*, indicating an important role of this metabolite for this response. The other indolic mutants tested are impacted in several distinct branches of the indolic pathway, such as GS and 4-OH-ICN but accumulate wild-type levels of camalexin, and thus activate SAR against *B. cinerea*. Metabolite profiles of these lines greatly vary among studies depending on the stimuli or pathogens employed. In response to *P. syringae* pv. *maculicola* (*Psm*) infection, the indolic GS *tmyb* mutant accumulates less camalexin, although it is not impaired in SAR activation (Stahl et al., 2016). Moreover, in response to UV light treatment, camalexin levels are reduced in *tmyb*, and this can be partially rescued when plants are exogenously supplied with IAOx or IAN (Frerigmann et al., 2015). However, in our study, *tmyb* accumulated wild-type levels of camalexin in response to *B. cinerea* and was not impacted in EE-induced SAR. These results indicate that MYB transcription factors are important for camalexin biosynthesis depending on the experimental conditions but that they are not required for EE-induced SAR activation.

WRKY33 is a transcription factor known for regulating expression of camalexin biosynthesis genes (Mao et al., 2011). In addition, WRKY33 has been shown to be involved in SAR activation, by regulating the expression of *ALD1* and therefore inducing the NHP pathway (Wang et al., 2018). Consequently, *wkry33* mutant is SAR-deficient (Wang et al., 2018). We show here that *wrky33* is highly susceptible to *B. cinerea* infection, like *cyp79b2/b3* double mutant and is impaired in EE-induced SAR. WRKY33 positively regulate genes involved in the biosynthesis of several indolic metabolites, including *CYP79B2/B3* (Barco and Clay, 2020), *AAO1* (Liu et al., 2015) as well as *FOX1* and *CYP82C2* (Barco et al., 2019). Consistently, *wrky33* accumulates reduced levels of camalexin and 4-OH-ICA, the aqueous degradation product of 4-OH-ICN, which has been shown to contribute to disease resistance towards *P. syringae* and *B. cinerea* (Rajniak et al., 2015). However, *fox1* and *cyp82c2-2* mutants, impaired

in ICN and 4-OH-ICN biosynthesis, respectively, were not more sensitive to *B. cinerea* infection (Fig. 1E), suggesting that they do not contribute to basal resistance, at least towards the *B. cinerea* strain used in our study. However, the amplitude of EE-induced SAR is slightly reduced in *cyp71a12*, *fox1* and *cyp82c2-2* mutants, which suggests a minor contribution of 4-OH-ICN in this response or simply depicts some variation among different experiments. Moreover, WRKY33 has been shown to negatively regulate SA and *wrky33* mutant accumulates more SA than Col-0 in response to *B. cinerea* (Birkenbihl et al., 2012), which could impact JA levels according to the hormonal crosstalk of these metabolites (Pieterse et al., 2009). Collectively, the loss of these responses in *wrky33* possibly contributes to its high susceptibility towards *B. cinerea*.

Interestingly, EE-induced SAR is abolished in *pad3-1*, which is defective in the last two steps of camalexin biosynthesis (Böttcher et al., 2009), indicating that camalexin is necessary for this response. Indeed, no other functions has been attributed for PAD3, suggesting that the only metabolite lacking in *pad3-1* is camalexin. However, since some P450 enzymes have been shown to catalyse several reactions from similar substrates, it is possible that PAD3 catalyses other reactions generating yet unknown metabolites. Camalexin is known to be detrimental to pathogenic bacteria and fungi by disrupting membrane integrity (Rogers et al., 1996). We confirm the high toxicity of camalexin towards our *B. cinerea* strain, as reported previously (Chassot et al., 2008). SAR is usually accompanied by a more robust activation of defence upon secondary infection, a process termed priming (Mauch-Mani et al., 2017). Priming of defence-relevant gene expression and metabolite accumulation has been observed in *Arabidopsis* previously (Návarová et al., 2012; Hilfiker et al., 2014). Pretreatment with EE leads to a significantly higher *PAD3* and *CYP71A13* expression upon secondary infection, which is abolished in the SAR-deficient *ald1* mutant (Supplemental Fig. 5). However, camalexin accumulation does not correlate with gene expression data, although a slight increase is observed 12 h post-infection (Fig. 2B). This effect was hardly reproducible in another experiment (Fig. 2C) and is not observed at later timepoints. Also, although significantly higher, camalexin levels 12 h post-infection are not substantial and probably not elevated enough to explain the reduced *B. cinerea* growth in systemic leaves of EE-treated plants.

Fungal pathogens usually detoxify antifungal compounds from various nature by active export or by biochemical modifications (Pedras and Ahiahonu, 2005). The *B. cinerea* gene *atrB* codes for an ABC transporter that can export phytoalexins such as camalexin and resveratrol from *Arabidopsis* and grapevine, respectively (de Waard et al., 2006; Stefanato et al., 2009). Interestingly, *atrB* is more induced when *B. cinerea* infects EE-treated plants compared to

untreated plants, and this is not observed in EE-treated *ald1* plants. This suggests that *B. cinerea* may face a higher concentration of antifungal metabolites in EE-treated Col-0 plants. However, this does not correlate with an increased accumulation of camalexin since it would have been detected in our protocol where whole infected leaves are harvested. Also, if *B. cinerea* can export more camalexin or other compounds and therefore be more virulent, lesion sizes would be logically higher in EE-treated plants, which was also not observed. On the other hand, reduced growth in EE-treated plants could potentially result from the high energy costs spent in such reactions. Finally, it is possible that *B. cinerea* exports through atrB other unidentified compounds without any functions for SAR activation and therefore results in increased expression of this gene. Pathogenic fungi can detoxify plant defence compounds by several ways that include hydrolysis, oxidation and reduction but also glycosylation (Pedras and Abdoli, 2017). It is possible that *B. cinerea* detoxifies excess amounts of camalexin resulting from an EE-induced priming, therefore escaping detection, but again, this would have most likely resulted in increased *B. cinerea* virulence, which is not observed. Alternatively, camalexin might be metabolised *in planta* to other non-quantified antifungal compounds and this process would be increased by EE pretreatment. Further experiments will be needed to test this hypothesis.

After being synthesised in the cytoplasm (Mucha et al., 2019), camalexin is secreted at the leaf surface by the transporters PEN3 and PDR12 for resistance against *B. cinerea* (He et al., 2019). We observe a slight priming of camalexin levels at the surface of leaves which is abolished in *ald1*, but the increase is very low and again, probably not high enough to explain growth reduction. Interestingly, it was found that the susceptibility of *pen3 pdr12* double mutant to *B. cinerea* was higher than *pad3* but comparable to *cyp79b2/b3* double mutant, suggesting that these transporters can mediate the secretion of other metabolites than camalexin, most likely other Trp-derived compounds (He et al., 2019). However, whether such unidentified metabolites are more abundant at the leaf surface and contribute to *B. cinerea* growth reduction during EE-induced SAR is unknown. It would be interesting to test whether EE-induced SAR is conserved in the *pen3 pdr12* double mutant.

Interestingly, we observe a substantial accumulation of camalexin in local leaves treated with EE for three and five days. This might explain the rapid reduction of *B. cinerea* growth observed in local EE-treated leaves (Chapter 1, Supplemental Fig. 2). Since EE treatment induces local accumulation of SA (Bruessow et al., 2010), one could reason that SA is involved in camalexin regulation. We however found that the SA-deficient mutant *ics1* accumulates wild-type levels of camalexin in response to *B. cinerea*, independently of EE pretreatment. This

confirms previous results showing that SA-deficient mutants normally accumulate camalexin (Nawrath and Métraux, 1999). Consistent with this, transcriptome analyses revealed that *PAD3* was still induced in systemic leaves of SAR-induced *ics1* plants (Bernsdorff et al., 2016). We showed in Chapter 1 (Fig. 3) that *npr1-1* and *npr1-1 npr4-4D* mutants, which are blocked in SA signalling, displayed enhanced basal susceptibility to *B. cinerea* and were impacted in EE-induced SAR. Furthermore, exogenous watering with the SAR signal NHP primed plants for enhanced camalexin accumulation, and this process was shown to be NPR1-dependent (Yildiz et al., 2021). In line with this, it would be interesting to quantify camalexin accumulation in response to EE pretreatment and *B. cinerea* infection in these mutants. Also, the pathogen-responsive kinases MPK3 and MPK6 have been shown to be involved in camalexin regulation (Ren et al., 2008). Interestingly, EE treatment also induces sustained activation of MPK3/6 (C. Gouhier-Darimont, unpublished), which could explain the high levels of camalexin in local EE-treated leaves.

In conclusion, we show that EE-induced SAR relies on camalexin, although accumulating levels in infected leaves or specifically at the leaf surface are not different between control and EE-pretreated plants. Furthermore, camalexin accumulates similar to Col-0 in the SAR-deficient mutants *ald1* and *ics1*, impaired in NHP and SA production, respectively. Thus, how EE pretreatment induces *B. cinerea* growth inhibition through camalexin remains to be elucidated.

MATERIALS AND METHODS

Plant and Insect Growth Conditions

Arabidopsis thaliana (Col-0) plants were sown in moist potting compost. After seed stratification for 2 days at 4°C, plants were grown for 4 weeks in growth chambers in short day conditions (10 h light/14 h dark), under 100 µmol m^{-2} s^{-1} of light, at 20-22°C and 65% relative humidity.

Lines used in this study: *ald1* (Návarová et al., 2012), *cyp71a12* (Millet et al., 2010), *cyp71a12 cyp71a13* (Müller et al., 2015), *cyp71b6 aao1* (Müller et al., 2019), *cyp79b2 cyp79b3* (Zhao et al., 2002), *cyp82c2-2* (Rajniak et al., 2015), *fox1* (Rajniak et al., 2015), *ics1* (*sid2-1* allele) (Nawrath and Métraux, 1999), *myb28 myb29* (Beekwilder et al., 2008), *myb34 myb51 myb122* (*tmyb*) (Frerigmann and Gigolashvili, 2014), *pad3-1* (Glazebrook and Ausubel, 1994), *wrky33* (Birkenbihl et al., 2012). All genotypes were in the Columbia (Col-0) background.

A population of the Large White butterfly *Pieris brassicae* was maintained on *Brassica oleracea* var. *gemmifera* in a greenhouse at 24°C and 65% relative humidity (Reymond et al., 2000).

Treatment with EE

For EE preparation, *P. brassicae* eggs were crushed with a pestle in Eppendorf tubes. After centrifugation (14,000 *g* for 3 min), the supernatant (EE) was collected and stored at -20°C. For application, 2 x 2 µl of EE were spotted under the surface of each of two leaves on at least 4-6 plants per independent experiment. Plants were treated 5 days before *B. cinerea* infection. Untreated plants were used as controls.

Culture of *B. cinerea*, Infection and Growth Assessment

B. cinerea strain BMM (Zimmerli et al., 2001) was grown on 1X PDA (Potato Dextrose Agar, 39 g l^{-1}, Difco) for 10-14 days in darkness at 23°C. Spores were harvested in water and filtered through wool placed in a 10 ml tip to remove hyphae. Spores were diluted in half-strength PDB (Potato Dextrose Broth, 12 g l^{-1}, Difco) to a concentration of 5 x 10^5 spores ml^{-1} for inoculation. One 5 µl droplet of spore suspension was deposited on the adaxial surface of two leaves per plant. Inoculated plants were kept under a water-sprayed transparent lid to maintain high

humidity in a growth chamber under dim light (around 2 µmol m^{-2} s^{-1}) during the whole time of infection. Lesion size measurements were made using ImageJ software version 2.0.0-rc-65/1.51u (http://imagej.nih.gov/ij). Data were analyzed with a linear mixed model fit by the restricted maximum likelihood (REML) algorithm (package 'lme4' in R, http://www.R-project.org).

Determination of Antifungal Activity

Camalexin (Glixx Laboratories, Hopkinton, USA) was dissolved in dimethylsulfoxide (DMSO) before use. Round plugs with a diameter of 0.5 cm were taken from a 7-days-old *B. cinerea* culture on 1X PDA and transferred to 6-well plates supplemented with different concentration of camalexin. Control plates contained 0.1% DMSO. For each concentration, radial growth of the fungal colony was measured on 2 plates (n=12) after 24 h of incubation at 23 °C in darkness. Mycelial growth inhibition (MGI) was calculated using the following formula: MGI % = [(C-T)/C] X 100 where C is the average colony diameter on control plates and T is the average colony diameter on treated plates. This experiment was done three times (different *B. cinerea* cultures).

Metabolite Analyses

For indolic compounds analyses, between 10 and 12 leaves (two leaves per plant) were harvested per time point and per treatment. Leaves were then pooled, frozen and ground with a pestle and mortar in liquid nitrogen. One hundred milligrams of frozen leaf powder were placed in a 1.5 ml Eppendorf tube and 500 µl of extraction buffer (80% methanol, 19.5% water and 0.5% formic acid) were added. After centrifugation (3 min at 14,000 *g*), 200 µl were disposed in vials. Camalexin was measured using ultra-high-performance liquid chromatography coupled to tandem mass spectrometry (UHPLC-MS/MS) according to (Balmer et al., 2018) and indolic GS by quadrupole time-of-flight mass spectrometry (UHPLC-QTOFMS) according to (Glauser et al., 2012).

For analysis of leaf surface camalexin, *B. cinerea*-infected or mock-treated leaves were immerged in 80% MeOH (2 mL/2 leaves) in 6-well plates and gently rotated for 30 sec. The solvent was collected in Eppendorf tubes and evaporated using a speed vac. The pellet was resuspended in 200 µl of 80% MeOH and transferred to vials for further LC-MS analysis. Quantification of camalexin was done according to (Balmer et al., 2018). Values were

normalized to the leaf surface and expressed as µg/cm^2. A total of 8 leaves (2 leaves from 4 plants) was used for each independent experiment.

The protocol for GS analysis was described earlier (Glauser et al., 2012). Briefly, 200 mg of frozen leaf powder were placed in 15 ml tube. Two ml of ice cold 70% MeOH and 15 µl of a solution of sinalbin (internal standard) were added immediately. After homogenization for 30 s, samples were incubated for 15 min at 80°C, centrifuged for 10 min at 3,500 x *g* and the supernatant was transferred to vials for UHPLC-QTOFMS measurements.

For all metabolite analyses, each experiment was done at least three times (different sampling dates).

Gene Expression Analysis

To monitor gene expression, total RNA was extracted using a ReliaPrep^{TM} RNA Tissue Miniprep System (Promega). For cDNA synthesis, 500 ng of total RNA was reverse-transcribed using M-MLV reverse transcriptase (Invitrogen) in a final volume of 15.25 µl. Each cDNA sample was generated in triplicate and diluted eightfold with water. Quantitative real-time PCR analysis was performed in a final volume of 20 µl containing 2 µl of cDNA, 0.2 µM of each primer, 0.03 µM of reference dye and 10 µl of Brilliant III Ultra Fast SYBR Green qPCR Master Mix (Agilent). Reactions were performed using an Mx3000P real-time PCR machine (Agilent) with the following program: 95°C for 3 min, then 40 cycles of 10 sec at 95°C and 20 sec at 60°C. Relative mRNA abundance of monitored genes was normalised to the housekeeping gene *SAND* (At2g28390). Primers used are listed in the Supplemental Table 1.

Statistical Analyses

Statistical analyses were performed using R software version 3.5.2 (http://www.R-project.org). Normal distribution and variance homogeneity of data were evaluated with Shapiro-Wilk and Levene's test, respectively. If not normal, data were log-transformed to ensure analyses with parametric tests.

To compare CTL vs EE within the same genotype in SAR bioassays, we used a linear mixed model fit by the restricted maximum likelihood (REML) algorithm (package "lme4" in R) using plant treatment as a fixed factor and experimental block as a random factor.

For metabolite quantifications, we used ANOVA with Tukey test for post-hoc comparison.

AUTHOR CONTRIBUTIONS

Esteban Alfonso performed the experiments and analysed data for all the figures except for Figure 2D that was performed by Elia Stahl.
For all metabolite quantifications (Fig. 2, 3A, 5, S1 and S2), Esteban Alfonso treated plants and extracted samples and Gaétan Glauser processed and analysed data.
Esteban Alfonso wrote the Chapter and Philippe Reymond reviewed and edited the text.

REFERENCES

Balmer A, Pastor V, Glauser G, Mauch-Mani B (2018) Tricarboxylates Induce Defense Priming Against Bacteria in Arabidopsis thaliana. Front Plant Sci **9**: 1221

Barco B, Clay NK (2020) Hierarchical and Dynamic Regulation of Defense-Responsive Specialized Metabolism by WRKY and MYB Transcription Factors. Front Plant Sci **10**: 1775

Barco B, Kim Y, Clay NK (2019) Expansion of a core regulon by transposable elements promotes Arabidopsis chemical diversity and pathogen defense. Nat Commun **10**: 3444

Bednarek P (2012) Chemical warfare or modulators of defence responses – the function of secondary metabolites in plant immunity. Current Opinion in Plant Biology **15**: 407–414

Bednarek P, Pislewska-Bednarek M, Svatos A, Schneider B, Doubsky J, Mansurova M, Humphry M, Consonni C, Panstruga R, Sanchez-Vallet A, et al (2009) A Glucosinolate Metabolism Pathway in Living Plant Cells Mediates Broad-Spectrum Antifungal Defense. Science **323**: 101–106

Bednarek P, Piślewska-Bednarek M, Ver Loren van Themaat E, Maddula RK, Svatoš A, Schulze-Lefert P (2011) Conservation and clade-specific diversification of pathogen-inducible tryptophan and indole glucosinolate metabolism in Arabidopsis thaliana relatives. New Phytologist **192**: 713–726

Bednarek P, Schneider B, Svatoš A, Oldham NJ, Hahlbrock K (2005) Structural Complexity, Differential Response to Infection, and Tissue Specificity of Indolic and Phenylpropanoid Secondary Metabolism in Arabidopsis Roots. Plant Physiology **138**: 1058–1070

Beekwilder J, van Leeuwen W, van Dam NM, Bertossi M, Grandi V, Mizzi L, Soloviev M, Szabados L, Molthoff JW, Schipper B, et al (2008) The Impact of the Absence of Aliphatic Glucosinolates on Insect Herbivory in Arabidopsis. PLoS ONE **3**: e2068

Bender J, Celenza JL (2009) Indolic glucosinolates at the crossroads of tryptophan metabolism. Phytochem Rev 13

Bernsdorff F, Döring A-C, Gruner K, Schuck S, Bräutigam A, Zeier J (2016) Pipecolic Acid Orchestrates Plant Systemic Acquired Resistance and Defense Priming via Salicylic Acid-Dependent and -Independent Pathways. Plant Cell **28**: 102–129

Birkenbihl RP, Diezel C, Somssich IE (2012) Arabidopsis WRKY33 Is a Key Transcriptional Regulator of Hormonal and Metabolic Responses toward *Botrytis cinerea* Infection. Plant Physiol **159**: 266–285

Bones A, Rossiter J (2006) The enzymic and chemically induced decomposition of glucosinolates. Phytochemistry **67**: 1053–1067

Böttcher C, Chapman A, Fellermeier F, Choudhary M, Scheel D, Glawischnig E (2014) The Biosynthetic Pathway of Indole-3-Carbaldehyde and Indole-3-Carboxylic Acid Derivatives in Arabidopsis. Plant Physiology **165**: 841–853

Böttcher C, Westphal L, Schmotz C, Prade E, Scheel D, Glawischnig E (2009) The Multifunctional Enzyme CYP71B15 (PHYTOALEXIN DEFICIENT3) Converts Cysteine-Indole-3-Acetonitrile to Camalexin in the Indole-3-Acetonitrile Metabolic Network of *Arabidopsis thaliana*. The Plant Cell **21**: 1830–1845

Bruessow F, Gouhier-Darimont C, Buchala A, Metraux J-P, Reymond P (2010) Insect eggs suppress plant defence against chewing herbivores: Insect eggs and plant defence. The Plant Journal **62**: 876–885

Chassot C, Buchala A, Schoonbeek H, Métraux J-P, Lamotte O (2008) Wounding of Arabidopsis leaves causes a powerful but transient protection against Botrytis infection. The Plant Journal 13

Clay NK, Adio AM, Denoux C, Jander G, Ausubel FM (2009) Glucosinolate Metabolites Required for an Arabidopsis Innate Immune Response. Science **323**: 95–101

Erb M, Kliebenstein DJ (2020) Plant Secondary Metabolites as Defenses, Regulators, and Primary Metabolites: The Blurred Functional Trichotomy. Plant Physiol **184**: 39–52

Ferrari S, Galletti R, Denoux C, De Lorenzo G, Ausubel FM, Dewdney J (2007) Resistance to *Botrytis cinerea* Induced in Arabidopsis by Elicitors Is Independent of Salicylic Acid, Ethylene, or Jasmonate Signaling But Requires *PHYTOALEXIN DEFICIENT3*. Plant Physiology **144**: 367–379

Ferrari S, Plotnikova JM, De Lorenzo G, Ausubel FM (2003) *Arabidopsis* local resistance to *Botrytis cinerea* involves salicylic acid and camalexin and requires *EDS4* and *PAD2* , but not *SID2* , *EDS5* or *PAD4*: *Local* Arabidopsis *response to* Botrytis. The Plant Journal **35**: 193–205

Frerigmann H, Gigolashvili T (2014) MYB34, MYB51, and MYB122 Distinctly Regulate Indolic Glucosinolate Biosynthesis in Arabidopsis thaliana. Molecular Plant **7**: 814–828

Frerigmann H, Glawischnig E, Gigolashvili T (2015) The role of MYB34, MYB51 and MYB122 in the regulation of camalexin biosynthesis in Arabidopsis thaliana. Front Plant Sci. doi: 10.3389/fpls.2015.00654

Frerigmann H, Piślewska-Bednarek M, Sánchez-Vallet A, Molina A, Glawischnig E, Gigolashvili T, Bednarek P (2016) Regulation of Pathogen-Triggered Tryptophan Metabolism in Arabidopsis thaliana by MYB Transcription Factors and Indole Glucosinolate Conversion Products. Molecular Plant **9**: 682–695

Geu-Flores F, Møldrup ME, Böttcher C, Olsen CE, Scheel D, Halkier BA (2011) Cytosolic γ-Glutamyl Peptidases Process Glutathione Conjugates in the Biosynthesis of Glucosinolates and Camalexin in *Arabidopsis*. The Plant Cell **23**: 2456–2469

Glauser G, Schweizer F, Turlings TCJ, Reymond P (2012) Rapid Profiling of Intact Glucosinolates in Arabidopsis Leaves by UHPLC-QTOFMS Using a Charged Surface Hybrid Column: Rapid Profiling of Intact Glucosinolate in Arabidopsis by Uhplc-Qtofms. Phytochem Anal **23**: 520–528

Glawischnig E (2007) Camalexin. Phytochemistry **68**: 401–406

Glawischnig E, Hansen BG, Olsen CE, Halkier BA (2004) Camalexin is synthesized from indole-3-acetaldoxime, a key branching point between primary and secondary metabolism in Arabidopsis. Proceedings of the National Academy of Sciences **101**: 8245–8250

Glazebrook J, Ausubel FM (1994) Isolation of phytoalexin-deficient mutants of Arabidopsis thaliana and characterization of their interactions with bacterial pathogens. Proceedings of the National Academy of Sciences **91**: 8955–8959

Hagemeier J, Schneider B, Oldham NJ, Hahlbrock K (2001) Accumulation of soluble and wall-bound indolic metabolites in Arabidopsis thaliana leaves infected with virulent or avirulent Pseudomonas syringae pathovar tomato strains. Proceedings of the National Academy of Sciences **98**: 753–758

Halkier BA, Gershenzon J (2006) BIOLOGY AND BIOCHEMISTRY OF GLUCOSINOLATES. Annu Rev Plant Biol **57**: 303–333

He Y, Xu J, Wang X, He X, Wang Y, Zhou J, Zhang S, Meng X (2019) The Arabidopsis Pleiotropic Drug Resistance Transporters PEN3 and PDR12 Mediate Camalexin Secretion for Resistance to *Botrytis cinerea*. Plant Cell **31**: 2206–2222

Hilfiker O, Groux R, Bruessow F, Kiefer K, Zeier J, Reymond P (2014) Insect eggs induce a systemic acquired resistance in Arabidopsis. Plant J **80**: 1085–1094

Hull AK, Vij R, Celenza JL (2000) Arabidopsis cytochrome P450s that catalyze the first step of tryptophan-dependent indole-3-acetic acid biosynthesis. Proceedings of the National Academy of Sciences **97**: 2379–2384

Kettles GJ, Drurey C, Schoonbeek H, Maule AJ, Hogenhout SA (2013) Resistance of *A rabidopsis thaliana* to the green peach aphid, *M yzus persicae* , involves camalexin and is regulated by micro RNA s. New Phytol **198**: 1178–1190

Khare D, Choi H, Huh SU, Bassin B, Kim J, Martinoia E, Sohn KH, Paek K-H, Lee Y (2017) *Arabidopsis* ABCG34 contributes to defense against necrotrophic pathogens by mediating the secretion of camalexin. Proc Natl Acad Sci USA **114**: E5712–E5720

Klein AP, Anarat-Cappillino G, Sattely ES (2013) Minimum Set of Cytochromes P450 for Reconstituting the Biosynthesis of Camalexin, a Major *Arabidopsis* Antibiotic. Angew Chem Int Ed **52**: 13625–13628

Koprivova A, Schuck S, Jacoby RP, Klinkhammer I, Welter B, Leson L, Martyn A, Nauen J, Grabenhorst N, Mandelkow JF, et al (2019) Root-specific camalexin biosynthesis controls the plant growth-promoting effects of multiple bacterial strains. Proc Natl Acad Sci USA **116**: 15735–15744

Kosaka A, Pastorczyk M, Piślewska-Bednarek M, Nishiuchi T, Ono E, Suemoto H, Ishikawa A, Frerigmann H, Kaido M, Mise K, et al (2021) Tryptophan-derived metabolites and BAK1 separately contribute to Arabidopsis postinvasive immunity against Alternaria brassicicola. Sci Rep **11**: 1488

Little D, Gouhier-Darimont C, Bruessow F, Reymond P (2007) Oviposition by Pierid Butterflies Triggers Defense Responses in Arabidopsis. Plant Physiol **143**: 784–800

Liu S, Kracher B, Ziegler J, Birkenbihl RP, Somssich IE (2015) Negative regulation of ABA signaling by WRKY33 is critical for Arabidopsis immunity towards Botrytis cinerea 2100. eLife **4**: e07295

Mao G, Meng X, Liu Y, Zheng Z, Chen Z, Zhang S (2011) Phosphorylation of a WRKY Transcription Factor by Two Pathogen-Responsive MAPKs Drives Phytoalexin Biosynthesis in *Arabidopsis*. Plant Cell **23**: 1639–1653

Mauch-Mani B, Baccelli I, Luna E, Flors V (2017) Defense Priming: An Adaptive Part of Induced Resistance. Annu Rev Plant Biol **68**: 485–512

Mikkelsen MD, Hansen CH, Wittstock U, Halkier BA (2000) Cytochrome P450 CYP79B2 from Arabidopsis Catalyzes the Conversion of Tryptophan to Indole-3-acetaldoxime, a Precursor of Indole Glucosinolates and Indole-3-acetic Acid. Journal of Biological Chemistry **275**: 33712–33717

Millet YA, Danna CH, Clay NK, Songnuan W, Simon MD, Werck-Reichhart D, Ausubel FM (2010) Innate Immune Responses Activated in *Arabidopsis* Roots by Microbe-Associated Molecular Patterns. The Plant Cell **22**: 973–990

Mitreiter S, Gigolashvili T (2021) Regulation of glucosinolate biosynthesis. Journal of Experimental Botany **72**: 70–91

Mucha S, Heinzlmeir S, Kriechbaumer V, Strickland B, Kirchhelle C, Choudhary M, Kowalski N, Eichmann R, Hueckelhoven R, Grill E, et al (2019) The formation of a camalexin-biosynthetic metabolon. Plant Cell tpc.00403.2019

Müller R, de Vos M, Sun JY, Sønderby IE, Halkier BA, Wittstock U, Jander G (2010) Differential Effects of Indole and Aliphatic Glucosinolates on Lepidopteran Herbivores. J Chem Ecol **36**: 905–913

Müller TM, Böttcher C, Glawischnig E (2019) Dissection of the network of indolic defence compounds in Arabidopsis thaliana by multiple mutant analysis. Phytochemistry **161**: 11–20

Müller TM, Böttcher C, Morbitzer R, Götz CC, Lehmann J, Lahaye T, Glawischnig E (2015) TRANSCRIPTION ACTIVATOR-LIKE EFFECTOR NUCLEASE-Mediated Generation and Metabolic Analysis of Camalexin-Deficient *cyp71a12 cyp71a13* Double Knockout Lines. Plant Physiology **168**: 849–858

Nafisi M, Goregaoker S, Botanga CJ, Glawischnig E, Olsen CE, Halkier BA, Glazebrook J (2007) *Arabidopsis* Cytochrome P450 Monooxygenase 71A13 Catalyzes the Conversion of Indole-3-Acetaldoxime in Camalexin Synthesis. The Plant Cell **19**: 2039–2052

Návarová H, Bernsdorff F, Döring A-C, Zeier J (2012) Pipecolic Acid, an Endogenous Mediator of Defense Amplification and Priming, Is a Critical Regulator of Inducible Plant Immunity. Plant Cell **24**: 5123–5141

Nawrath C, Métraux J-P (1999) Salicylic Acid Induction–Deficient Mutants of Arabidopsis Express PR-2 and PR-5 and Accumulate High Levels of Camalexin after Pathogen Inoculation. 13

Parisy V, Poinssot B, Owsianowski L, Buchala A, Glazebrook J, Mauch F (2007) Identification of PAD2 as a γ-glutamylcysteine synthetase highlights the importance of glutathione in disease resistance of Arabidopsis: Glutathione deficiency and Arabidopsis disease resistance. The Plant Journal **49**: 159–172

Pastorczyk M, Kosaka A, Piślewska-Bednarek M, López G, Frerigmann H, Kułak K, Glawischnig E, Molina A, Takano Y, Bednarek P (2020) The role of CYP 71A12 monooxygenase in pathogen-triggered tryptophan metabolism and Arabidopsis immunity. New Phytol **225**: 400–412

Pedras MSC, Abdoli A (2017) Pathogen inactivation of cruciferous phytoalexins: detoxification reactions, enzymes and inhibitors. RSC Adv **7**: 23633–23646

Pedras MSC, Ahiahonu PWK (2005) Metabolism and detoxification of phytoalexins and analogs by phytopathogenic fungi. Phytochemistry **66**: 391–411

Pedras MSC, Yaya EE, Glawischnig E (2011) The phytoalexins from cultivated and wild crucifers: Chemistry and biology. Nat Prod Rep **28**: 1381

Piasecka A, Jedrzejczak-Rey N, Bednarek P (2015) Secondary metabolites in plant innate immunity: conserved function of divergent chemicals. New Phytol **206**: 948–964

Pieterse CMJ, Leon-Reyes A, Van der Ent S, Van Wees SCM (2009) Networking by small-molecule hormones in plant immunity. Nat Chem Biol **5**: 308–316

Qiu J-L, Fiil BK, Petersen K, Nielsen HB, Botanga CJ, Thorgrimsen S, Palma K, Suarez-Rodriguez MC, Sandbech-Clausen S, Lichota J, et al (2008) Arabidopsis MAP kinase 4 regulates gene expression through transcription factor release in the nucleus. EMBO J **27**: 2214–2221

Rajniak J, Barco B, Clay NK, Sattely ES (2015) A new cyanogenic metabolite in Arabidopsis required for inducible pathogen defence. Nature **525**: 376–379

Ren D, Liu Y, Yang K-Y, Han L, Mao G, Glazebrook J, Zhang S (2008) A fungal-responsive MAPK cascade regulates phytoalexin biosynthesis in Arabidopsis. Proceedings of the National Academy of Sciences **105**: 5638–5643

Reymond P, Weber H, Damond M, Farmer EE (2000) Differential Gene Expression in Response to Mechanical Wounding and Insect Feeding in Arabidopsis. 14

Rogers EE, Glazebrook J, Ausubel FM (1996) Mode of Action of the *Arabidopsis thaliana* Phytoalexin Camalexin and Its Role in *Arabidopsis-Pathogen* Interactions. MPMI **9**: 748

Sanchez-Vallet A, Ramos B, Bednarek P, López G, Piślewska-Bednarek M, Schulze-Lefert P, Molina A (2010) Tryptophan-derived secondary metabolites in Arabidopsis thaliana confer non-host resistance to necrotrophic Plectosphaerella cucumerina fungi: Arabidopsis non-host resistance to necrotrophic fungi. The Plant Journal no-no

Schlaeppi K, Abou-Mansour E, Buchala A, Mauch F (2010) Disease resistance of Arabidopsis to Phytophthora brassicae is established by the sequential action of indole glucosinolates and camalexin: Glucosinolates and camalexin in disease resistance. The Plant Journal **62**: 840–851

Schlaeppi K, Bodenhausen N, Buchala A, Mauch F, Reymond P (2008) The glutathione-deficient mutant pad2-1 accumulates lower amounts of glucosinolates and is more susceptible to the insect herbivore Spodoptera littoralis. The Plant Journal 13

Schuhegger R, Nafisi M, Mansourova M, Petersen BL, Olsen CE, Svatoš A, Halkier BA, Glawischnig E (2006) CYP71B15 (PAD3) Catalyzes the Final Step in Camalexin Biosynthesis. Plant Physiology **141**: 1248–1254

Stahl E, Bellwon P, Huber S, Schlaeppi K, Bernsdorff F, Vallat-Michel A, Mauch F, Zeier J (2016) Regulatory and Functional Aspects of Indolic Metabolism in Plant Systemic Acquired Resistance. Molecular Plant **9**: 662–681

Stahl E, Brillatz T, Ferreira Queiroz E, Marcourt L, Schmiesing A, Hilfiker O, Riezman I, Riezman H, Wolfender J-L, Reymond P (2020) Phosphatidylcholines from Pieris brassicae eggs activate an immune response in Arabidopsis. eLife **9**: e60293

Stefanato FL, Abou-Mansour E, Buchala A, Kretschmer M, Mosbach A, Hahn M, Bochet CG, Métraux J-P, Schoonbeek H (2009) The ABC transporter BcatrB from *Botrytis cinerea* exports camalexin and is a virulence factor on *Arabidopsis thaliana*. The Plant Journal **58**: 499–510

Thomma BPHJ, Nelissen I, Eggermont K, Broekaert WF (1999) Deficiency in phytoalexin production causes enhanced susceptibility of Arabidopsis thaliana to the fungus Alternaria brassicicola. Plant J **19**: 163–171

Tsuji J, Jackson EP, Gage DA, Hammerschmidt R, Somerville SC (1992) Phytoalexin Accumulation in *Arabidopsis thaliana* during the Hypersensitive Reaction to *Pseudomonas syringae* pv *syringae*. Plant Physiol **98**: 1304–1309

de Waard MA, Andrade AC, Hayashi K, Schoonbeek H, Stergiopoulos I, Zwiers L-H (2006) Impact of fungal drug transporters on fungicide sensitivity, multidrug resistance and virulence. Pest Manag Sci **62**: 195–207

Wang Y, Schuck S, Wu J, Yang P, Döring A-C, Zeier J, Tsuda K (2018) A MPK3/6-WRKY33-ALD1-Pipecolic Acid Regulatory Loop Contributes to Systemic Acquired Resistance. Plant Cell **30**: 2480–2494

Windram O, Madhou P, McHattie S, Hill C, Hickman R, Cooke E, Jenkins DJ, Penfold CA, Baxter L, Breeze E, et al (2012) Arabidopsis Defense against Botrytis cinerea: Chronology and Regulation Deciphered by High-Resolution Temporal Transcriptomic Analysis. Plant Cell **24**: 3530–3557

Xu J, Meng J, Meng X, Zhao Y, Liu J, Sun T, Liu Y, Wang Q, Zhang S (2016) Pathogen-Responsive MPK3 and MPK6 Reprogram the Biosynthesis of Indole Glucosinolates and Their Derivatives in Arabidopsis Immunity[OPEN]. 19

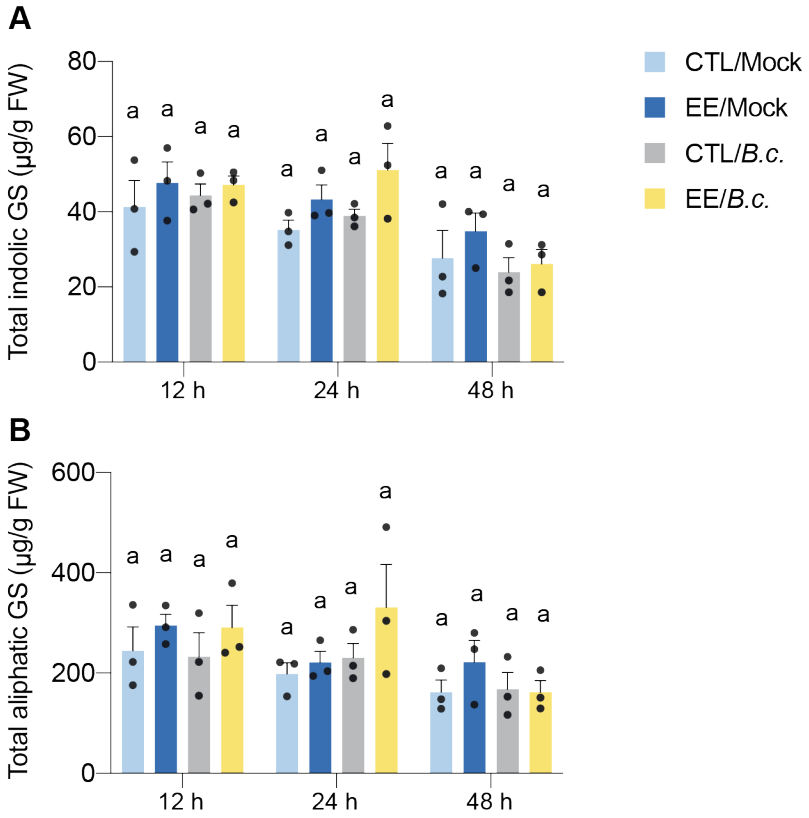
Yildiz I, Mantz M, Hartmann M, Zeier T, Kessel J, Thurow C, Gatz C, Petzsch P, Köhrer K, Zeier J (2021) The mobile SAR signal N-hydroxypipecolic acid induces NPR1-dependent transcriptional reprogramming and immune priming. Plant Physiology kiab166

Zhao Y, Hull AK, Gupta NR, Goss KA, Alonso J, Ecker JR, Normanly J, Chory J, Celenza JL (2002) Trp-dependent auxin biosynthesis in Arabidopsis: involvement of cytochrome P450s CYP79B2 and CYP79B3. 13

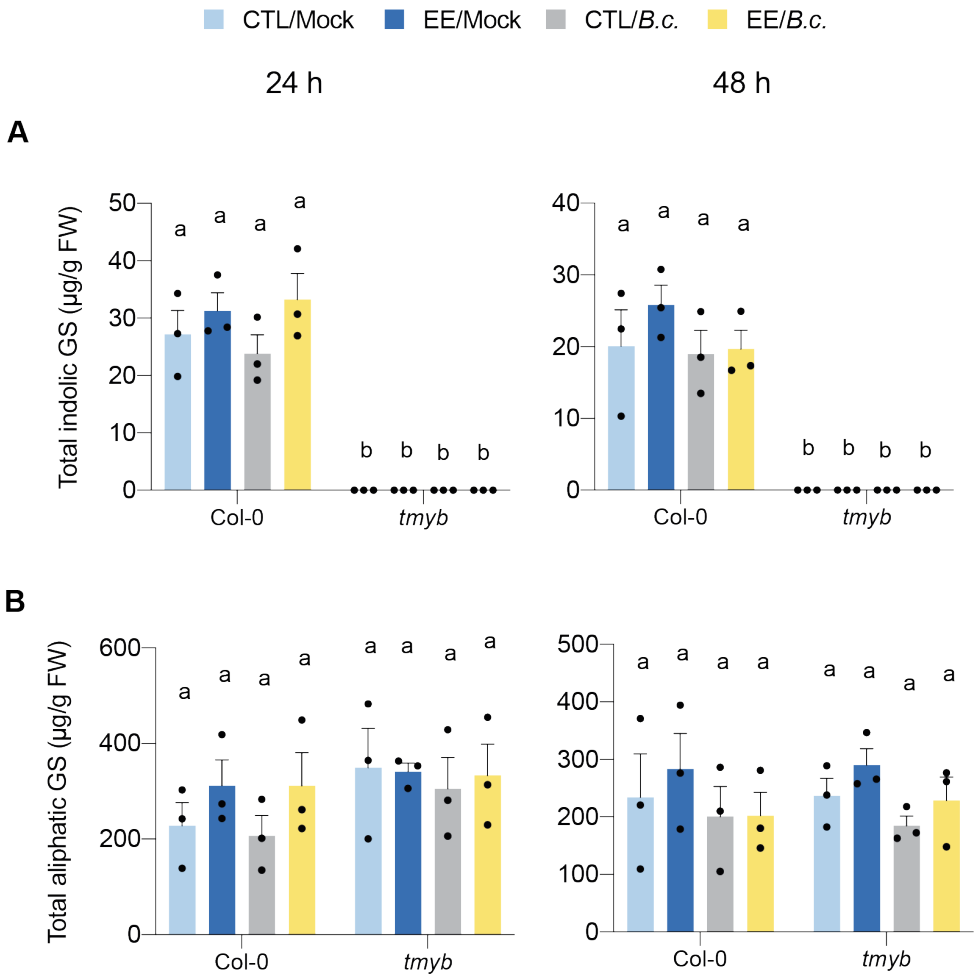
Zhou N, Tootle TL, Glazebrook J (1999) Arabidopsis PAD3, a Gene Required for Camalexin Biosynthesis, Encodes a Putative Cytochrome P450 Monooxygenase. Proc Natl Acad Sci USA **11**: 2419–2428

Zimmerli L, Métraux J-P, Mauch-Mani B (2001) β-Aminobutyric Acid-Induced Protection of Arabidopsis against the Necrotrophic Fungus Botrytis cinerea. Plant Physiology **126**: 517–523

SUPPLEMENTAL DATA



Supplemental Figure 1. Time-course of glucosinolates accumulation.
Local leaves were left untreated (CTL) or pretreated with *P. brassicae* EE for 5 days (EE) and distal leaves were further inoculated with *B. cinerea* spore suspension (*B.c.*) or a mock solution (Mock) for 12 h, 24 h and 48 h. Indolic glucosinolates (GS) (**A**) and aliphatic GS (**B**) were measured in distal leaves of Col-0 plants. Means ± SE of three independent experiments are shown (n = 10-12 leaves per experiment). Different letters indicate significant difference at $P < 0.05$ (ANOVA followed by Tukey's Honest Significant Difference test).



Supplemental Figure 2. Glucosinolates accumulation.
Local leaves were left untreated (CTL) or pretreated with *P. brassicae* EE for 5 days (EE) and distal leaves were further inoculated with *B. cinerea* spore suspension (*B.c.*) or a mock solution (Mock) for 24 h and 48 h. Indolic glucosinolates (GS) (**A**) and aliphatic GS (**B**) were measured in distal leaves of Col-0 and *tmyb* plants. Means ± SE of three independent experiments are shown (n = 10-12 leaves per experiment). Different letters indicate significant difference at $P < 0.05$ (ANOVA followed by Tukey's Honest Significant Difference test). *tmyb* = *myb34 myb51 myb122*.

Supplemental Table 1. List of primers used for RT-qPCR.

Gene name	Gene ID	Primers ID	Sequences (5'-3')	Reference
SAND	At2g28390	SAND-Fw	AACTCTATGCAGCATTTGATCCACT	Gouhier-Darimont et al. (2013)
		SAND-Rv	TGATTGCATATCTTTATCGCCATC	
CYP71A13	At2g30770	CYP71A13-Fw	ATGCCCCGGGATAAATCTT	
		CYP71A13-Rv	GAGAAAACATGTTACACAACC	
PAD3	At3g26830	PAD3-Fw	GTCAAGGAGACATTAAGGTTAC	
		PAD3-Rv	ACCCATCGCATAAACGTTGAC	
WRKY33	At2g38470	WRKY33-Fw	TACGAAGGGAAACACAACCA	Birkenbihl et al. (2012)
		WRKY33-Rv	AAGGCCCGGTATTAGTGTTG	
BcatrB		BcatrB-Fw	TCTAACCCCGCTGAACACAT	Stefanato et al. (2009)
		BcatrB-Rv	TTGCGGTAAATGGCTACGTT	
BcEF1b		BcEF1b-Fw	GCTGCCAAGTCTGTTGTCACA	Stefanato et al. (2009)
		BcEF1b-Rv	CAATGCTACCATGTCGGTCTC	

CHAPTER 3

Pieris brassicae EE treatment induces systemic accumulation of indole-3-carbaldehyde and indole-3-carboxylic acid glucose conjugates

Esteban Alfonso[1], Elia Stahl[1], Gaétan Glauser[2], Olivier Hilfiker[1], Raphaël de Matos[3], Sandrine Gerber[3] and Philippe Reymond[1]

[1]Department of Plant Molecular Biology, University of Lausanne, Switzerland
[2]Neuchâtel Platform of Analytical Chemistry, University of Neuchâtel, Switzerland
[3]Institute of Chemical Sciences and Engineering, Ecole Polytechnique Fédérale de Lausanne, Switzerland

ABSTRACT

Indole-3-carboxylic acid (ICA) and indole-3-carbaldehyde (ICHO) are Trp-derived metabolites that accumulate in response to pathogen attack. These compounds accumulate in their free forms but also as hydroxylated and glucose conjugates derivatives. We show here that *Pieris brassicae* egg extract (EE) pretreatment induces a substantial accumulation of ICHO and ICA glucose conjugates in systemic leaves. This accumulation is completely abolished in the pipecolic acid (Pip)-deficient mutant *ald1*, and exogenous Pip supplementation does not restore the *ald1* phenotype. We identified potential UDP-glycosyltransferases regulated by ALD1 that might be important for the conjugation of ICHO and ICA. Moreover, EE-induced systemic accumulation of these compounds still occurs in *cyp71b6 aao1* and *cyp71a12 cyp71a13* mutants, that are described as ICHO/ICA biosynthetic enzymes. Interestingly, accumulation of these conjugates is severely impacted in a *myb34 myb51 myb122* triple mutant, suggesting a role of one or several of these transcription factors in regulating these conjugations. We also show that ICHO and ICA exhibit direct antifungal activity towards *B. cinerea*, although ICA-Glc altered only mildly fungal growth.

INTRODUCTION

Brassicaceae plants have evolved specific Trp-derived metabolites with important roles in defence against herbivores and pathogens (Bednarek, 2012; Kettles et al., 2013; Maier et al., 2021). Trp is converted by CYP79B2 and CYP79B3 to indole-3-acetaldoxime (IAOx), the common precursor for indolic glucosinolates (GS), camalexin and indole-3-carboxylic acid (ICA) formation (Mikkelsen et al., 2000). Indolic GS are phytoanticipins that have been shown to be a feeding and oviposition stimulant for specialist insects, such as *Pieris brassicae*, but also to be deterrent to generalist chewing herbivores and pathogens (Bednarek et al., 2009; Hopkins et al., 2009). In *Arabidopsis*, indolic GS regulation is controlled by three MYB transcription factors, namely MYB34, MYB51 and MYB122, and a triple mutant (*tmyb*) is completely devoid of indolic GS (Frerigmann and Gigolashvili, 2014). The phytoalexin camalexin is induced by a large variety of pathogens and some abiotic stresses, such as high metals concentration and UV light (Glawischnig, 2007). Its biosynthesis starts from IAOx conversion to indole-3-acetonitrile (IAN), a reaction catalysed by the two monooxygenases CYP71A12 and CYP71A13. Then, IAN is further conjugated with glutathione and cysteine in multiple steps to dihydrocamalexin, which is finally converted to camalexin by PAD3 (Schuhegger et al., 2006; Nafisi et al., 2007; Böttcher et al., 2009). These enzymes physically interact to form a complex, termed metabolon, anchored to the membrane of the endoplasmic reticulum, allowing high flux of camalexin biosynthesis (Mucha et al., 2019).

In addition to camalexin, the IAN-derived metabolites indole-3-carbaldehyde (ICHO) and ICA accumulate in response to pathogen infection and abiotic stresses in *Arabidopsis* leaves and roots (Hagemeier et al., 2001; Bednarek et al., 2005; Forcat et al., 2010; Stahl et al., 2016). ICA has been shown to be rapidly esterified to cell wall in response to *Pseudomonas syringae* infection (Tan et al., 2004; Forcat et al., 2010). Co-expression analyses indicated that *CYP71B6* and *ARABIDOPSIS ALDEHYDE OXIDASE 1* (*AAO1*) genes are co-expressed with each other and with the camalexin biosynthetic genes *CYP71A13* and *PAD3* during pathogen infection (Böttcher et al., 2014). Expression of *CYP71B6* and *AAO1* in heterologous systems as well as the use of *Arabidopsis* knockout and overexpression lines allowed to uncover a contribution of these enzymes in ICHO and ICA biosynthesis from IAN *in vitro* and in response to silver nitrate *in planta* (Koiwai et al., 2000; Böttcher et al., 2014). In addition to its function in IAOx conversion to IAN (Klein et al., 2013), CYP71A12 also catalyses the formation of ICHO from IAN, similar to CYP71B6, although IAOx is the preferred substrate (Müller et al., 2015). In *cyp71a12/a13* double mutant, although camalexin levels are null, ICHO, ICA and their

derivatives still accumulate in response to silver nitrate and UV treatments (Müller et al., 2015), suggesting that multiple sources of IAN exist. Indolic GS degradation possibly constitutes a source of IAN and indole-3-carbinol, from which ICHO and ICA are produced, involving CYP71B6 and AAO1 (Kim et al., 2008; de Vos et al., 2008; Müller et al., 2015). ICHO and ICA accumulate in their free forms but also as methylated, hydroxylated or glycosylated derivatives (Böttcher et al., 2014). ICHO and ICA derivatives were initially identified in chemical complementation experiments where leaves of the *cyp79b2/b3* double mutant were exogenously supplied with IAN, ICHO and ICA and exposed to abiotic stresses. Derived metabolites were then identified by LC-MS (Böttcher et al., 2014). ICHO derivatives are mainly glycosylated and hydroxylated forms of ICHO. For ICA, methyl, glucosyl and malonylated glucosyl ester derivatives as well as aspartate conjugates were identified (Böttcher et al., 2014).

Biosynthesis of Trp-derived metabolites is triggered by the recognition of microbe-associated molecular patterns (MAMPs), such as the 22-amino-acid fragment of bacterial flagellin (flg22) (Clay et al., 2009; Millet et al., 2010). In addition to their function in indolic GS regulation, MYB34, MYB51 and MYB122 also contribute to other IAOx-derived metabolites regulation (Frerigmann et al., 2016). Upon flg22 treatment, *Arabidopsis* induced callose deposition in a MYB51-dependent manner (Clay et al., 2009). The *tmyb* mutant displayed constitutively reduced levels of 6-Glc-ICA and ICA-Glc, two ICA glycosylated derivatives, upon flg22 treatment (Frerigmann et al., 2016). Furthermore, in local leaves infected by *P. syringae* pv. *maculicola* (*Psm*), the *tmyb* mutant accumulated reduced levels of ICHO, ICA and camalexin (Stahl et al., 2016). Contrastingly, upon infection by the necrotrophic fungus *Plectospherella cucumerina*, *tmyb* accumulated higher levels of camalexin, 6-Glc-ICA and ICA-Glc compared to Col-0, indicating drastic differences between treatments (Frerigmann et al., 2016). An *Arabidopsis* mutant lacking specifically ICHO or ICA and their derivatives has not been described so far, making it difficult to assess the importance of these compounds in plant immunity (Müller et al., 2019). Nevertheless, when exogenously applied, ICA increased resistance to *P. cucumerina* by inducing a priming of callose deposition, thus restricting pathogen entry, but did not show direct antifungal activity towards this pathogen and was thus suggested to have a signalling or regulatory function *in planta* (Gamir et al., 2012; Gamir et al., 2014).

Indolic metabolism is also activated during systemic acquired resistance (SAR). Following local *Psm* infection, ICHO and ICA accumulated in uninfected systemic tissue, and this was found to be dependent on a functional SAR signalling, involving the NHP and SA pathways as well as functional CYP79B2/B3 and MYB34/51/122 (Stahl et al., 2016). However,

cyp79b2/b3 and *tmyb* mutants were still able to activate SAR against *Psm*, indicating that the systemic accumulation of ICHO and ICA is a consequence and not the cause of SAR activation. Moreover, exogenously applied ICA has just a marginal effect on *Arabidopsis* resistance against *Psm* (Stahl et al., 2016).

We showed in Chapter 2 that EE-induced SAR against *Botrytis cinerea* is dependent on camalexin accumulation. Indolic mutants lacking camalexin are impaired in EE-induced SAR activation, compared to mutants accumulating camalexin but impaired in other branches of indolic metabolism. Here, we focus on other Trp-derived metabolites, ICHO, ICA as well as their glycosylated conjugates, which accumulate to high levels in systemic leaves following local EE treatment. We show that this accumulation occurs in a Pip-independent manner, although it requires ALD1. We also quantify these metabolites in various mutants of the indolic metabolism and show that their accumulation is not correlated with EE-induced SAR against *B. cinerea*.

RESULTS

ICHO/ICA conjugates accumulate in response to EE in an *ALD1*-dependent manner

We quantified ICHO and ICA as well as their glucose-conjugated derivatives (Fig. 1) (hereafter termed as conjugates) in distal leaves after *P. brassicae* EE pretreatment and/or *B. cinerea* infection. ICA levels increased only in response to *B. cinerea* infection in Col-0 and in the Pip-deficient *ald1* mutant, with no further accumulation after EE pretreatment (Fig. 2A). Furthermore, ICA accumulated locally but not distally in response to EE treatment (Supplemental Fig. 1). ICHO accumulation was more variable, although levels seemed to also increase after *B. cinerea* infection (Fig. 2B). Strikingly, we observed a substantial accumulation of ICA conjugates in distal leaves after EE pretreatment 24 h post-inoculation (hpi) (Fig. 2C). Indeed, total accumulation of the main conjugated forms of ICA (ICA-Glc, 6-HO-ICA-Glc, 6-Glc-ICA and 6-Glc-ICA-Glc; (Böttcher et al., 2014)) reached ca. 8 µg/g FW of ICA equivalents, a value 10 x higher than corresponding ICA levels (Fig. 2C). Each conjugate approximately accumulated 2-fold more in EE pretreated samples (EE/Mock and EE/*B.c.*) compared to controls (CTL/Mock and CTL/*B.c.*). Notably, EE-induced systemic accumulation of ICA conjugates was completely abolished in *ald1,* suggesting that this is a Pip-regulated response. Similarly, systemic total accumulation of ICHO conjugates (4-Glc-ICHO and 5-Glc-ICHO) after EE pretreatment reached levels around 10 x higher than corresponding ICHO and

depended on ALD1 (Fig. 2D). Furthermore, these conjugates accumulated only weakly after *B. cinerea* infection, although a slight increase, yet not statistically different, was observed in *ald1* 48 hpi (Fig. 2C).

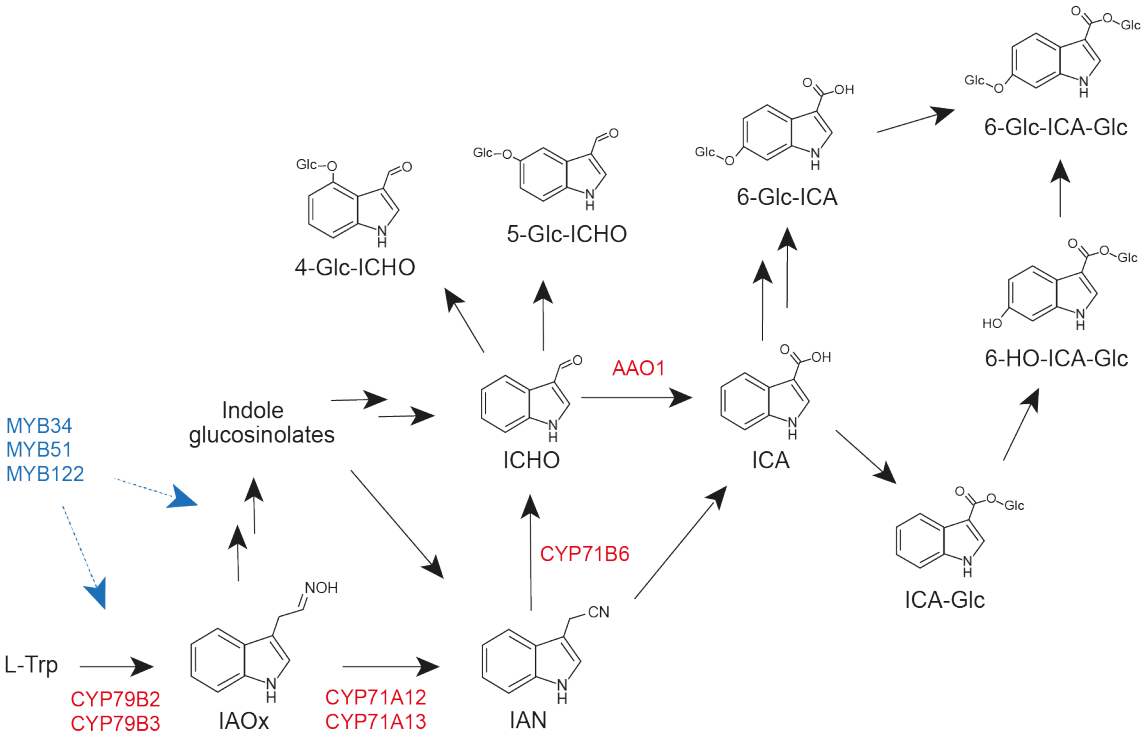


Figure 1. Tryptophan-derived metabolites.
Simplified scheme of biosynthesis of Trp-derived ICHO, ICA and their conjugates. Position of biosynthetic (red) and regulatory (blue) genes tested in this study. Several arrows indicate multiple steps. L-Trp, tryptophan; IAOx, indole-3-acetaldoxime; IAN, indole-3-acetonitrile; ICHO, indole-3-carbaldehyde; ICA, indole-3-carboxylic acid; Glc, glucose.

To further confirm the role of Pip in this response, we attempted to restore EE-induced accumulation of ICA and ICHO conjugates in *ald1* by exogenous Pip application. Plants were left untreated or pretreated with EE for 5 days and one day prior *B. cinerea* infection, a 1 mM Pip solution was pipetted onto the soil of Col-0 and *ald1* for uptake via the root system (Návarová et al., 2012). We then quantified ICA and ICHO conjugates 24 h after *B. cinerea* infection. Surprisingly, Pip watering did not complement EE-induced accumulation of the conjugates in *ald1* (Fig. 3). It also did not further increase levels of the conjugates in EE-treated Col-0. Nevertheless, EE-induced priming of *PR1* gene expression was restored in *ald1* supplemented with Pip (Supplemental Fig. 2), indicating that the treatment was efficient. These results suggest that ALD1, rather than Pip, is required for the EE-induced accumulation of these metabolites.

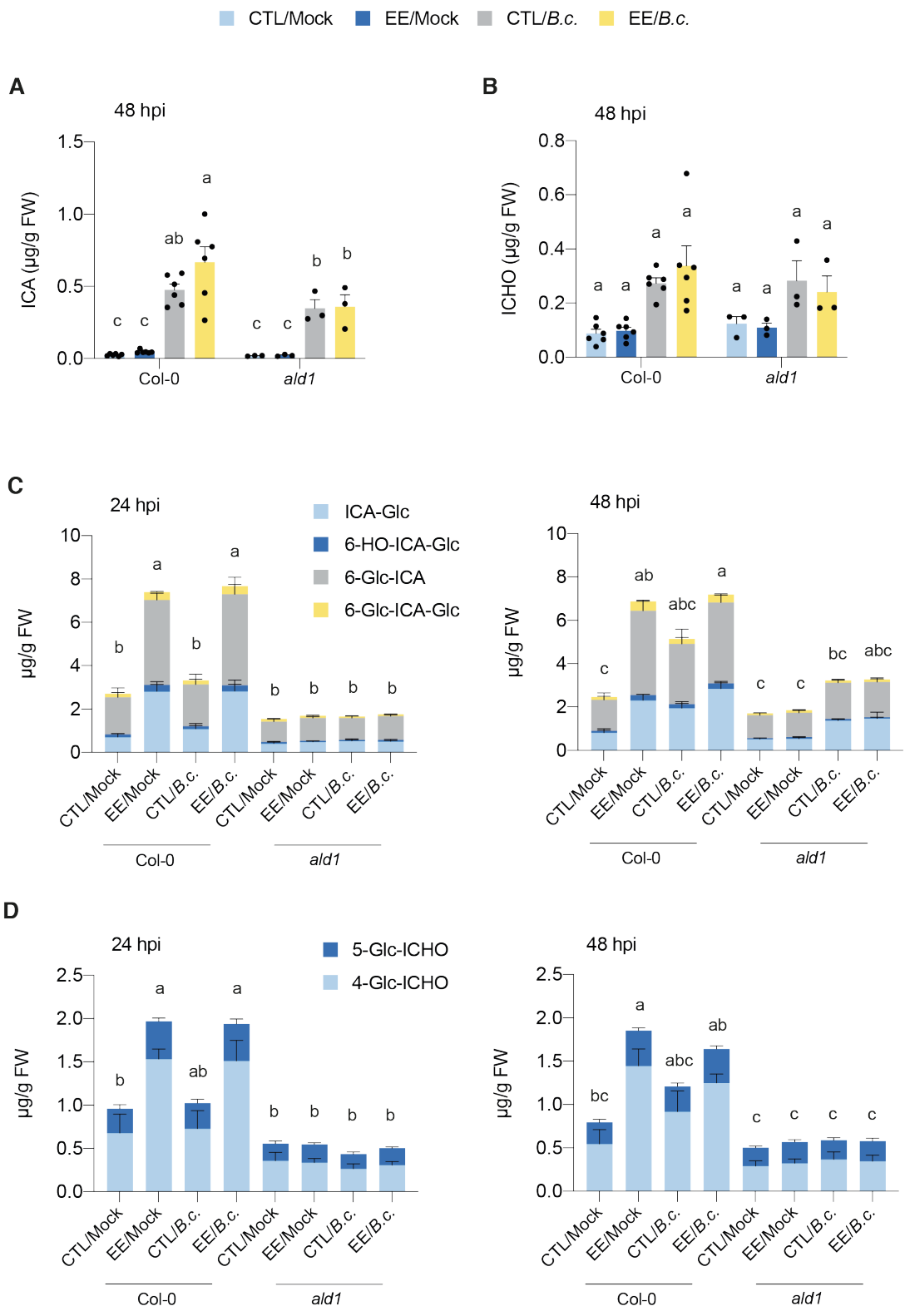


Figure 2. ICHO, ICA and conjugates accumulation in response to *P. brassicae* EE and *B. cinerea* treatments. Local leaves were left untreated (CTL) or pretreated with *P. brassicae* EE for 5 days (EE) and distal leaves were further inoculated with *B. cinerea* spore suspension (*B.c.*) or a mock solution (Mock) for 24 h and 48 h. ICA (**A**), ICHO (**B**), ICA conjugates (**C**) and ICHO conjugates (**D**) levels were measured in distal leaves. Means ± SE of six (Col-0) and three (ald1) independent experiments are shown (n = 10-12 leaves per experiment). Different letters indicate significant difference at P< 0.05 (ANOVA followed by Tukey's Honest Significant Difference test). Hpi, hours post-inoculation.

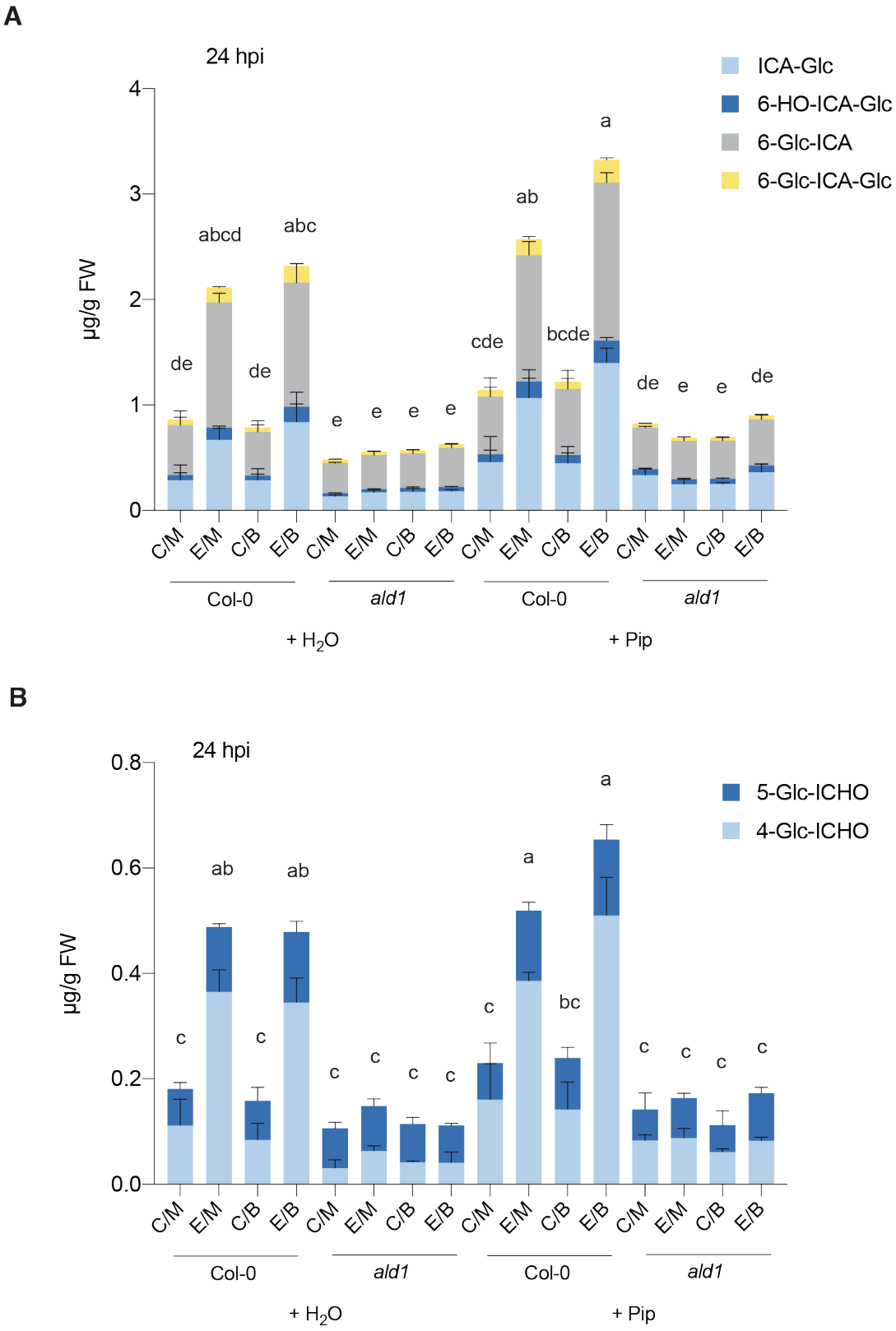


Figure 3. Pip watering does not restore ICHO/ICA conjugates accumulation in *ald1*.
Local leaves were left untreated (C) or pretreated with P. brassicae EE for 5 days (E) and distal leaves were further inoculated with B. cinerea spore suspension (B) or a mock solution (M) for 24 h. Water or 1 mM pipecolic acid (Pip) was applied to the soil one day prior inoculation. ICA conjugates (**A**) and ICHO conjugates (**B**) levels were measured 24 hours post-inoculation (hpi). Means ± SE of three independent experiments are shown (n = 10-12 leaves per experiment). Different letters indicate significant difference at $P < 0.05$ (ANOVA followed by Tukey's Honest Significant Difference test).

Pip and SA biosynthesis are commonly regulated and metabolically act together to orchestrate SAR (Hartmann and Zeier, 2019). We quantified SA levels in Col-0 and *ald1* after EE pretreatment and/or *B. cinerea* infection. We found that SA levels accumulated in distal leaves of EE-treated Col-0 plants and this accumulation was completely abolished in *ald1*, at both 24 and 48 hpi (Fig. 4A). Intriguingly, this pattern was the same than for ICA and ICHO conjugates accumulation. We thus decided to quantify ICA and ICHO conjugates in the SA-deficient mutant *ics1*, to investigate the role of SA in EE-induced accumulation of these conjugates. Although EE-induced accumulation of the conjugates in Col-0 was not as high as in previous experiments, *ics1* mutant was not depleted of the conjugates and a similar trend than in Col-0 was observable in EE-treated plants, at least 48 h after *B. cinerea* infection (Fig. 4B-C). Together, these results show that systemic EE-induced accumulation of ICA and ICHO conjugates are Pip- and SA-independent but seem to be ALD1-dependent.

Glucose conjugation of ICHO and ICA requires the action of glycosyltransferases. In line with the abolished EE-induced systemic accumulation of ICHO/ICA conjugates in *ald1*, we decided to monitor expression of genes coding for uridine diphosphate (UDP)-glycosyltransferases (UGT) in Col-0 and *ald1*. Currently, 120 UGTs have been characterised in the *Arabidopsis* genome (Li et al., 2001). To select appropriate candidates, we searched for UGTs that were expressed in local and distal leaves 5 days after *P. brassicae* oviposition and treatment with EE using available RNA sequencing data (Stahl et al., 2020). The complete list of UGTs induced upon oviposition and EE treatment is summarised in the Supplemental Table 1. In local leaves, 17 UGTs were significantly induced following *P. brassicae* oviposition in and 25 after EE treatment. In distal leaves, only one gene, *UGT73D1*, was induced after EE treatment and none after oviposition. We decided to select UGTs that were commonly expressed following these two treatments, which lowered the list to 16 candidates (Fig. 5A). We then monitored expression of these 16 UGTs by qPCR, looking for some candidates whose expression was altered in *ald1* compared to Col-0 in local and distal leaves following EE treatment for 5 days. Out of the 16 candidates, only five were significantly less expressed locally and/or distally in *ald1* compared to Col-0 (Fig. 5B). These candidates could represent potential UGTs able to glycosylate ICHO/ICA in distal leaves, along with unknown UGTs, since ALD1 has a minor role in local accumulation of the conjugates (Supplemental Fig. 3).

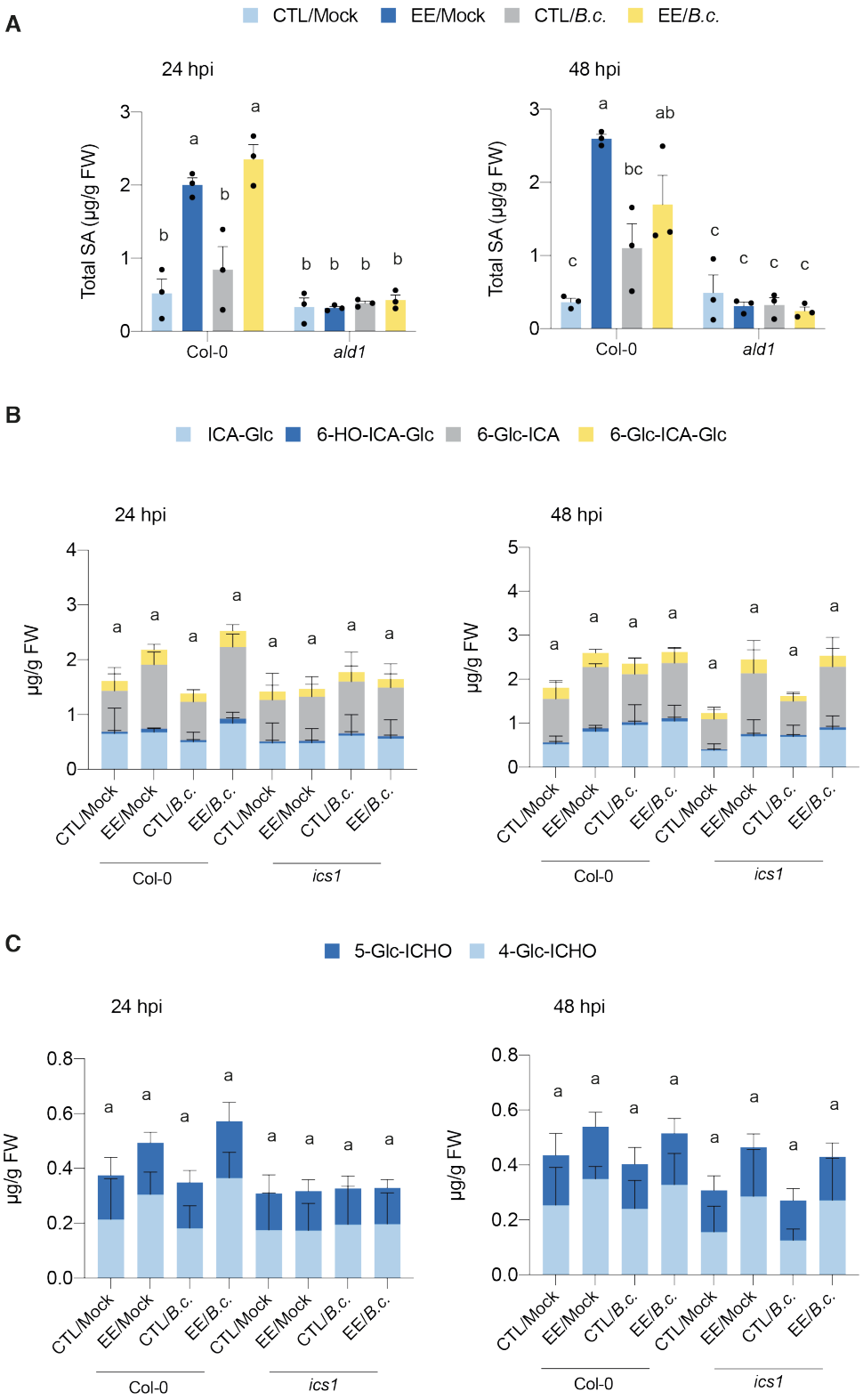


Figure 4. The SA pathway is not required for ICHO/ICA conjugates accumulation.
Local leaves were left untreated (CTL) or pretreated with *P. brassicae* EE for 5 days (EE) and distal leaves were further inoculated with *B. cinerea* spore suspension (*B.c.*) or a mock solution (Mock) for 24 h and 48 h. (**A**) Total SA was measured in Col-0 and *ald1* at 24 and 48 hpi. (**B**) ICA conjugates were quantified in Col-0 and *ics1* at 24 and 48 hpi. (**C**) ICHO conjugates were quantified in Col-0 and *ics1* at 24 and 48 hpi. Means ± SE of three independent experiments are shown (n = 10-12 leaves per experiment). Different letters indicate significant difference at $P < 0.05$ (ANOVA followed by Tukey's Honest Significant Difference test). Dots indicate individual values.

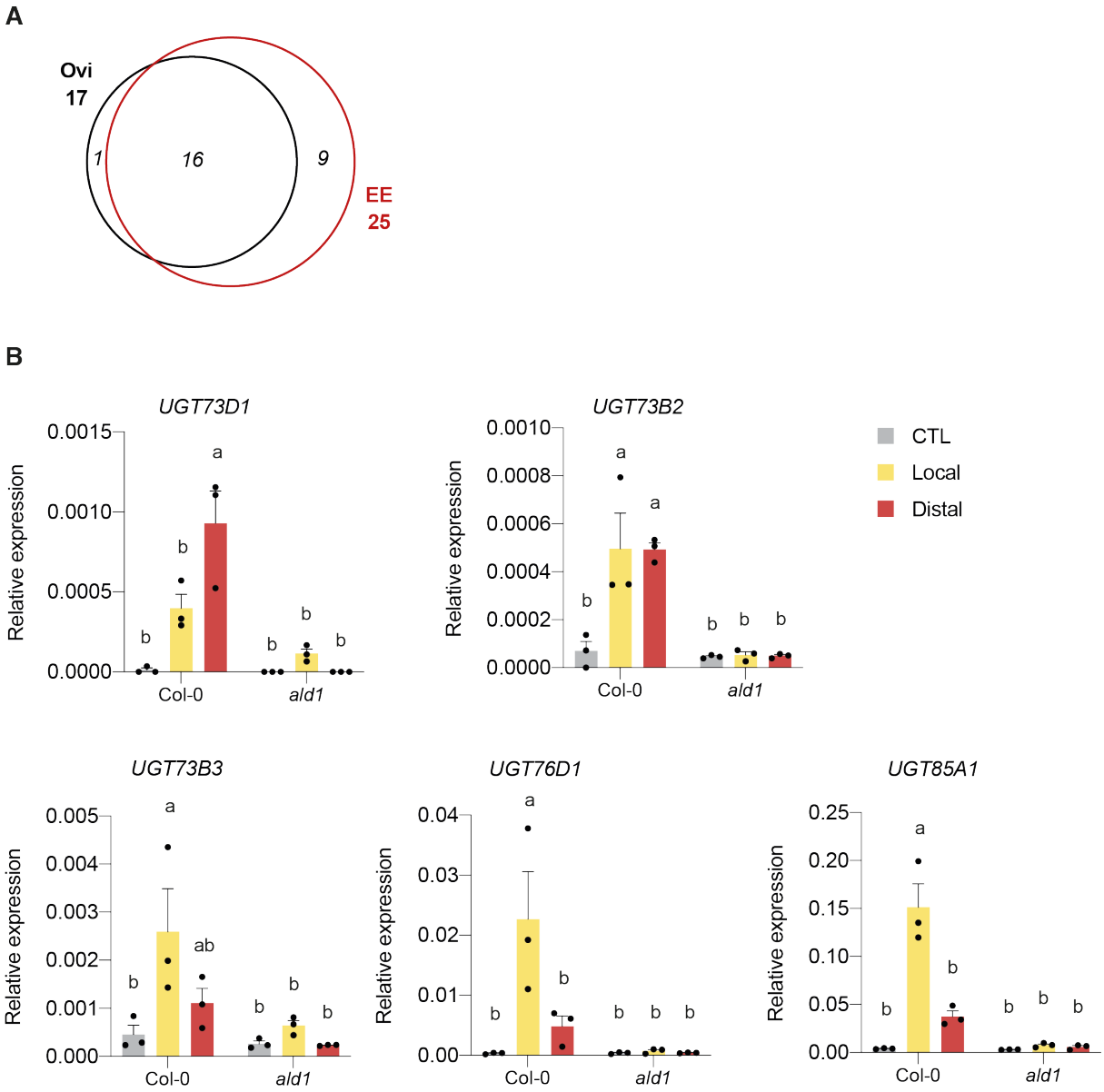


Figure 5. *P. brassicae* oviposition and EE treatment induce expression of genes encoding UDP-glycosyltransferases.
(**A**) Venn diagram showing oviposition (Ovi)- and EE-induced genes coding for UGTs. (**B**) Plant genotypes were left untreated or treated with EE for 5 days. Expression of genes coding for five UGTs was monitored in untreated (CTL), EE-treated leaves (Local) and leaves distal from EE-treated leaves (Distal). Means ± SE of three independent experiments are shown (n = 10-12 leaves per experiment). Different letters indicate significant difference at $P < 0.05$ (ANOVA followed by Tukey's Honest Significant Difference test). Dots indicate individual values.

Since *B. cinerea* infection triggered ICA and ICHO accumulation and EE pretreatment led to a substantial accumulation of ICHO/ICA conjugates, we hypothesized that these metabolites could inhibit *B. cinerea* growth. We thus tested whether ICA, ICHO and ICA-Glc displayed antifungal activity against *B. cinerea*. *B. cinerea* growth was monitored *in vitro* on plates supplemented with 25 or 50 µg/ml of these compounds. We compared it to camalexin, which was shown to fully inhibit *B. cinerea* growth (Ferrari et al., 2003). Interestingly, we found that 50 µg/ml of ICHO and ICA led to 40% inhibition of fungal growth, whereas ICA-Glc inhibited around 20% (Fig. 6). This suggests that glucose conjugation reduces the antifungal activity of ICA. Unfortunately, we were unable to test other ICA and ICHO conjugates since these compounds are not commercially available.

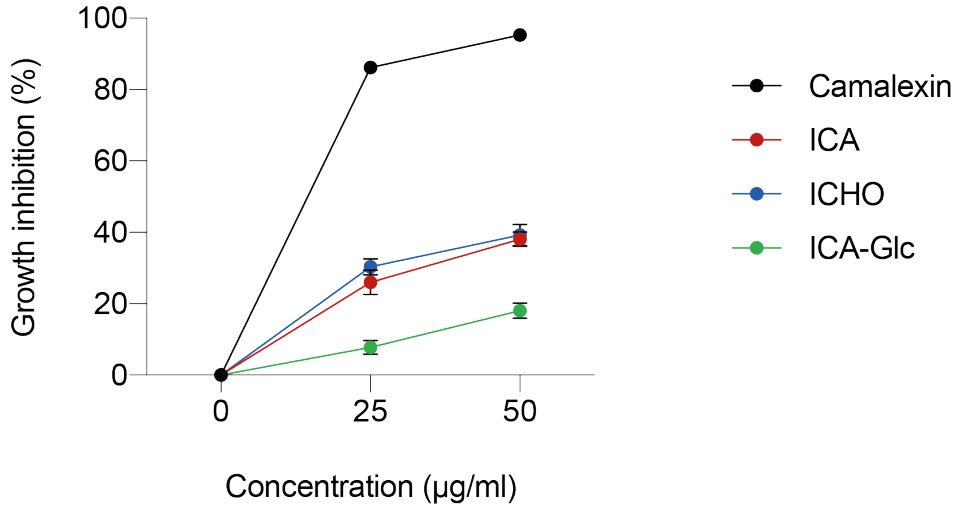


Figure 6. ICA-Glc displays lower toxicity to *B. cinerea* than ICA.
Radial growth of a *B. cinerea* colony growing on PDA plates supplemented with different concentrations of camalexin, ICA, ICHO and ICA-Glc was measured after 24 h of incubation. Means ± SE of one experiment are shown (n = 12).

EE-induced accumulation of ICHO/ICA conjugates in indolic mutants

The role of ICHO, ICA and their derivatives in plant immunity remains difficult to study, since no mutants lacking specifically these compounds have been described so far (Böttcher et al., 2014; Müller et al., 2019). We thus decided to investigate the involvement of CYP71A12, CYP71A13, CYP71B6, AAO1 and the MYB transcription factors MYB34, MYB51 and MYB122 in the biosynthesis of ICHO/ICA conjugates following EE treatment and/or *B. cinerea* infection. As expected, ICHO, ICA and their conjugates all derived from IAOx, as the *cyp79b2/b3* double mutant failed to accumulate these metabolites in response to any treatments (Fig. 7). We then quantified these compounds in the *cyp71a12/a13* double mutant (*71a12/a13*) and found that they accumulated significantly more after EE pretreatment and not after *B. cinerea* infection (Fig. 8A-B), unlike their precursors ICHO and ICA, which accumulated only in response to *B. cinerea* infection (Supplemental Fig. 4A).

We found a similar EE-induced accumulation of ICHO and ICA conjugates in the *cyp71b6 aao1* double mutant (*71b6 aao1*) (Fig. 8C-D). In addition, we found a higher level (~ 2.5 x more) of 5-Glc-ICHO in response to EE pretreatment (EE/Mock and EE/*B.c.*) in *cyp71b6 aao1* compared to Col-0 (Fig. 8D). We also observed this increased accumulation of 5-Glc-ICHO in systemic leaves of EE-treated plants and in response to *B. cinerea* infection in the *cyp71b6 aao1 cyp71a12 cyp71a13* quadruple mutant (abbreviated *b6/aao1/a12a13*), which also accumulated ICHO and ICA conjugates, although total amounts were slightly lower than in previous experiments in both Col-0 and *b6/aao1/a12a13* (Supplemental Fig. 5C-D). Interestingly, ICA levels 24 hpi were severely reduced in *b6/aao1/a12a13*, while ICHO levels were not different than Col-0 (Supplemental Fig. 5A-B).

Finally, we quantified ICHO/ICA conjugates in the *myb34 myb51 myb122* triple mutant (*tmyb*), which is completely devoid of indolic GS (Frerigmann and Gigolashvili, 2014) under the same experimental conditions. We found that levels of ICA conjugates were dramatically reduced in every condition tested in *tmyb* compared to Col-0 (Fig. 8E). ICA-Glc and 6-Glc-ICA-Glc could not be detected and only a small fraction of 6-HO-ICA-Glc and 6-Glc-ICA was still accumulating, with no further accumulation following EE treatment. Interestingly, levels of both ICHO conjugates were completely absent in *tmyb* (Fig. 8F).

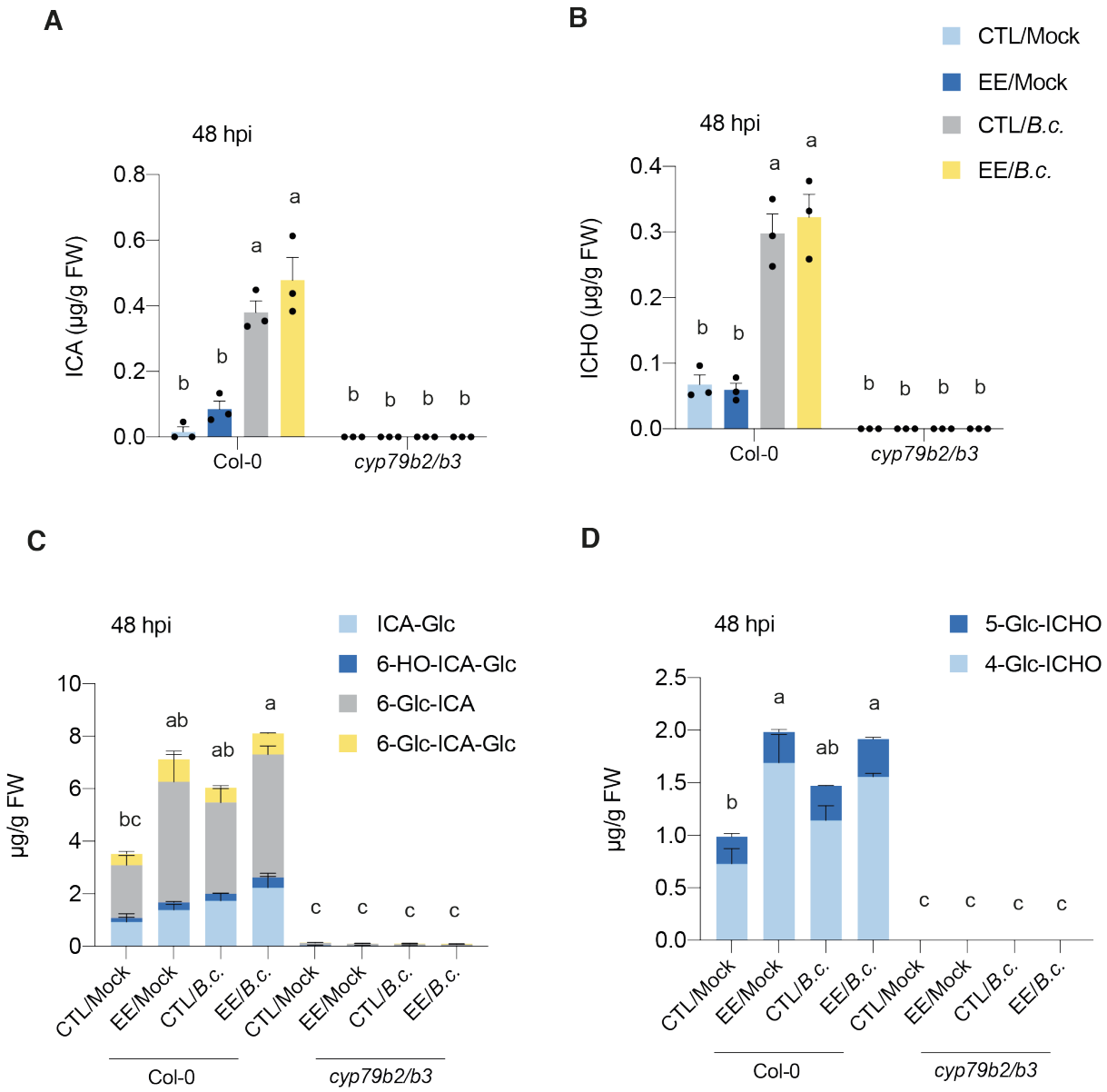


Figure 7. ICHO, ICA and their conjugates derive from IAOx.
Local leaves were left untreated (CTL) or pretreated with *P. brassicae* EE for 5 days (EE) and distal leaves were further inoculated with *B. cinerea* spore suspension (*B.c.*) or a mock solution (Mock) for 48 h. ICA (**A**), ICHO (**B**), ICA conjugates (**C**) and ICHO conjugates (**D**) levels were measured in distal leaves. Means ± SE of three independent experiments are shown (n = 10-12 leaves per experiment). Different letters indicate significant difference at $P < 0.05$ (ANOVA followed by Tukey's Honest Significant Difference test). Dots indicate individual values.

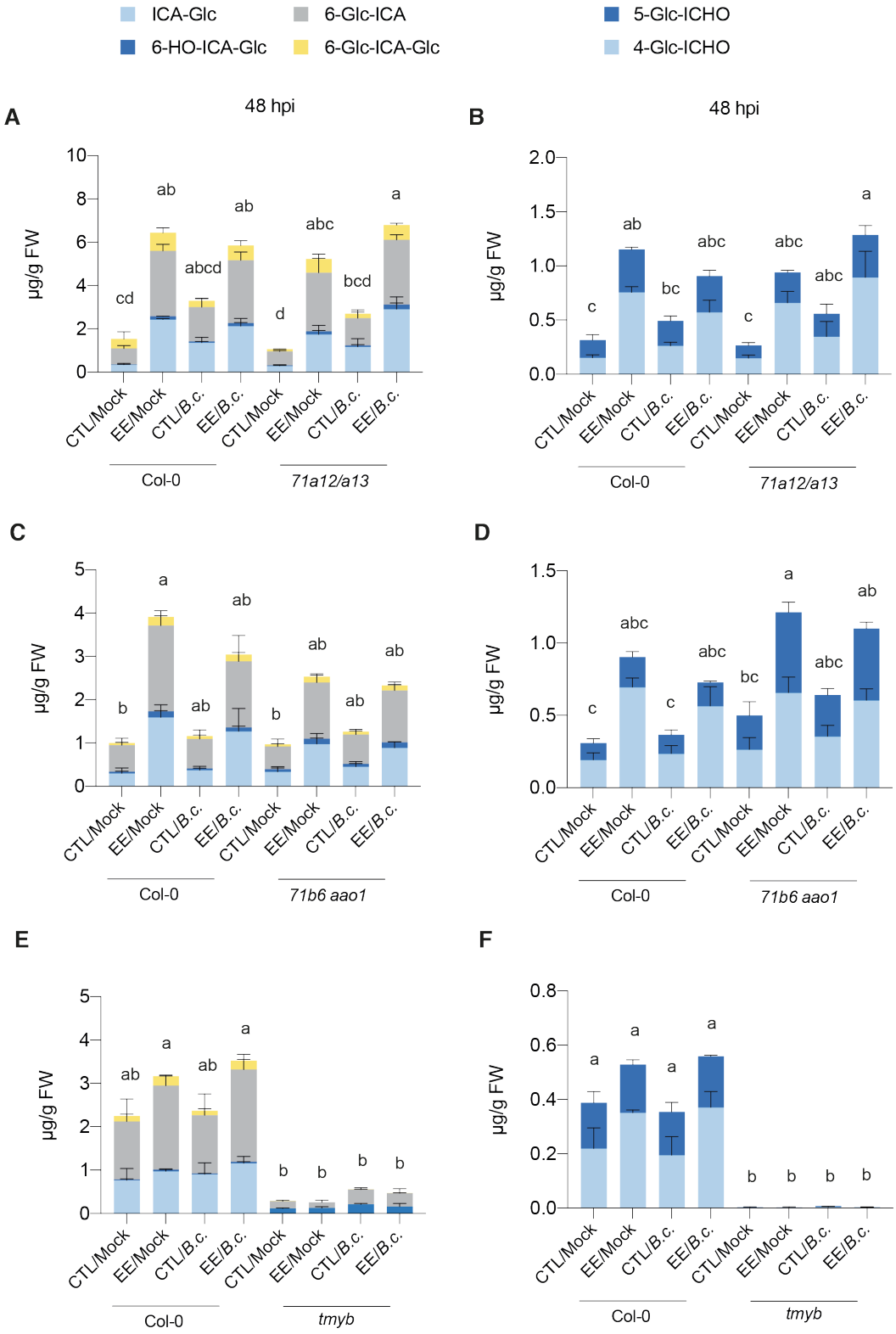


Figure 8. ICHO/ICA conjugates accumulation in indolic mutants.
Local leaves were left untreated (CTL) or pretreated with *P. brassicae* EE for 5 days (EE) and distal leaves were further inoculated with *B. cinerea* spore suspension (*B.c.*) or a mock solution (Mock) for 48 h. ICA and ICHO conjugates levels were measured in distal leaves of *cyp71a12 cyp71a13* (*71a12/a13*) double mutant (**A-B**), *cyp71b6 aao1* (*71b6 aao1*) double mutant (**C-D**) and *myb34 myb51 myb122* triple mutant (*tmyb*) (**E-F**). Means ± SE of three independent experiments are shown (n = 10-12 leaves per experiment). Different letters indicate significant difference at P< 0.05 (ANOVA followed by Tukey's Honest Significant Difference test).

DISCUSSION

EE-induced accumulation of ICHO/ICA conjugates requires ALD1 but not Pip

Many studies have focused on the role of camalexin deterring pathogenic fungi, but the involvement of ICAs in plant defence has been largely neglected. Upon challenge with *Psm*, ICHO, ICA and the GS-derived indole-3-ylmethylamine were the only indolic compounds to accumulate both in local bacteria-inoculated leaves and in leaves distal from inoculation (Stahl et al., 2016). Although levels of free ICA do not increase significantly in distal leaves from EE-treated plants, EE induces ICA accumulation in local leaves. However, important amounts of ICHO and ICA conjugates accumulate in leaves distal from EE treatment. They may serve as storage forms of antifungal compounds. Upon challenge with *B. cinerea* and other pathogens, these conjugates may be rapidly hydrolysed into free ICHO and ICA and display their inhibitory effect. Indeed, we showed that ICHO and ICA reduce about 40 % of *B. cinerea* mycelial growth when tested *in vitro*. Remarkably, ICA-Glc reduces twice as less *B. cinerea* growth, indicating that glucose conjugation diminishes the antifungal activity of ICA. This is in line with one of the main functions of glycosylation, which is to inactivate and detoxify harmful metabolites (Gachon et al., 2005). However, whether ICA-Glc is more toxic to other pathogens is possible but remains unknown. Indeed, plant-pathogen interactions are known to be influenced by variations in pathogen sensitivity and metabolite production in the host (Kliebenstein et al., 2005). The other ICA conjugates as well as ICHO conjugates were not tested since they were not commercially available. Synthesis of these compounds and testing their toxicity in *in vitro* assays should help answering these questions.

EE-induced systemic accumulation of ICHO and ICA conjugates is completely abolished in the Pip-deficient *ald1* mutant. Surprisingly, Pip watering is not sufficient to restore the conjugates accumulation in *ald1*. Exogenously applied Pip by watering has been shown to be taken up by the root system and is capable to restore the SAR-deficient phenotype of *ald1* by priming of defence gene expression and the accumulation of defence regulatory metabolites (Návarová et al., 2012; Bernsdorff et al., 2016). We also show that EE-induced priming of *PR1* expression is restored in *ald1* after Pip watering, which suggests a role for ALD1 enzyme in this response. ALD1 is a chloroplast-localised aminotransferase essential for local disease resistance to pathogens and SAR establishment (Song et al., 2004b; Cecchini et al., 2015). Upon bacterial infection, it transfers the amino group of lysine to acceptor molecules, generating an intermediate that is subsequently reduced to generate Pip and therefore inducing SAR (Ding et

al., 2016; Hartmann et al., 2017). However, ALD1 can use other substrates for transamination reactions *in vitro*, such as methionine and arginine, although lysine is the preferred substrate (Song et al., 2004a). A function of ALD1 in regulating UGTs is surprising and has not been described so far. However, we found that EE treatment induces the expression of several UGTs, whose five of them being expressed in an *ALD1*-dependent manner. Using an *ALD1*-overexpressing *Arabidopsis* line, it has been shown that ALD1 can generate at least one or several non-Pip metabolites capable of inducing early defence responses (Cecchini et al., 2015). Whether other ALD1-generated metabolites have a function on the regulation of UGTs remains unknown. However, it is possible that EE-induced *ALD1* expression leads to the production of other non-Pip metabolites regulating UGTs with potential functions on ICHO/ICA conjugates production. Several *Arabidopsis* UGTs, including UGT74D1 and UGT84B1, were shown to glycosylate auxin (indole-3-acetic acid, IAA) in *in vitro* assays (Jackson et al., 2001; Jin et al., 2013). Even though IAA is structurally similar to ICA, UGT74D1 shows only trace activity towards ICA (Jin et al., 2013). It would be interesting to express individually the five EE-induced UGTs in *Saccharomyces cerevisiae*, which naturally produces high amount of UDP-glucose (Oka and Jigami, 2006) and screen for ICHO and ICA conjugates production after supplementing the culture media with ICHO and ICA, respectively. Alternatively, quantification of ICHO/ICA conjugates in response to EE treatment in knockout lines of these UGTs would also be necessary to test their involvement in such glycosylation reactions. We propose a model indicating how EE treatment induces ICHO/ICA conjugates formation, which might help to visualise this complex pathway (Fig. 9).

So far, these results indicate that EE-induced accumulation of ICHO/ICA conjugates is not important for SAR establishment against *B. cinerea*. We previously showed that Pip watering of EE-treated *ald1* plants is sufficient to restore SAR (Chapter 1, Fig. 4B). Here, we show that Pip watering does not restore systemic accumulation of ICHO/ICA conjugates in EE-treated *ald1* plants, which strongly shows that accumulation of these conjugates is not linked with EE-induced SAR against *B. cinerea*. SA is another important regulator of EE-induced SAR (Hilfiker et al., 2014) and the accumulation of conjugates in *ics1* as well as the absence of EE-induced SAR in this mutant indicate again that these ICHO/ICA conjugates are not crucial for SAR establishment against *B. cinerea*. However, whether these metabolites are important for EE-induced SAR against other pathogens is unknown and would be interesting to investigate.

EE-induced accumulation of ICHO/ICA conjugates is not abolished in indolic mutants

We show here that levels of ICHO and ICA conjugates increase in distal leaves of EE-treated *cyp71b6 aao1* and *cyp71a12/a13* double mutants, as well as in the *cyp71b6 aao1 cyp71a12/a13* quadruple mutant. These enzymes were initially described to be part of the biosynthetic pathway of ICHO and ICA, and consequently their derivatives. This indicates that some alternative routes to these metabolites exist in *Arabidopsis* and more work will be needed to identify the enzymes responsible for their formation. Around 250 members of CYP450 enzymes were identified in *Arabidopsis* genome (Bak et al., 2011) and some of them have broad substrate specificity. Also, some reactions can occur spontaneously in plant cells, which makes the metabolome unpredictable from the genome. CYP71A12 and CYP71A13 show 89 % identity on the amino acid level and the genes are located as tandem copies on chromosome 2 (Müller et al., 2015). These two enzymes catalyse the formation of IAN from IAOx (Klein et al., 2013), but CYP71A12 can also catalyse the formation of ICHO from IAN, similar to CYP71B6, although IAN is the preferred substrate (Müller et al., 2015). This shows the possible multifunctionality of CYP450 enzymes depending on the substrate available. A third homolog, CYP71A18 shares more than 85 % homology with these two members and its biological function remains unclear, but it might play a role in synthesising ICA since constitutive accumulation of ICA conjugates is not impacted in *cyp71a12/a13* double mutant (Müller et al., 2015; Pastorczyk et al., 2020). Also, various IAN sources have been described, not only from CYP71A12/A13 functions. Indeed, indolic GS breakdown products can release indole-3-carbinol and IAN, which could then be processed by other CYP450 enzymes to generate ICHO and ICA, and therefore possibly contributing to conjugates formation (Kim et al., 2008; de Vos et al., 2008; Müller et al., 2015). A recent study used all combinations of *cyp71b6*, *aao1*, *cyp71a12* and *cyp71a13* mutants to decipher the network of indolic compounds in response to abiotic stresses. In this study, levels of ICA-Glc are reduced in *cyp71b6*, *cyp71a12/a13* and in the *cyp71b6 cyp71a12/a13* triple mutant in response to silver nitrate and UV treatments (Müller et al., 2019). Similarly, levels of 6-HO-ICA-Glc and 6-Glc-ICA are reduced in *cyp71a12/a13* (Müller et al., 2019). However, we do not observe such reductions in *cyp71b6 aao1*, *cyp71a12/a13* and in *b6 aao1 a12/a13* quadruple mutant upon EE treatment. Comparisons between this study and ours is difficult to assess, mainly due to the nature of stresses applied (silver nitrate and UV versus EE and *B. cinerea* infection). Interestingly, we observe an increase of 5-Glc-ICHO in *cypb6 aao1* and in *b6 aao1 a12/a13* in response to EE treatment, which is also observed in the same mutants challenged with silver nitrate and UV. This is correlated with

increased levels of ICHO in lines carrying the *aao1* mutation (Müller et al., 2019), even though no increased accumulation of ICHO is observed under our experimental conditions. Redirection of the network towards GS metabolism could justify such ICHO increases when ICHO/ICA biosynthetic pathway is blocked, and it would be interesting to verify this following EE treatment and/or *B. cinerea* infection in the *b6 aao1 a12/a13* quadruple mutant.

ICA-Glc and 6-Glc-ICA conjugates accumulate in *Arabidopsis* in response to infection by the necrotrophic fungal pathogens *P. cucumerina*, *Alternaria brassicicola* and *B. cinerea* (Bednarek et al., 2011; Pastorczyk et al., 2020; Kosaka et al., 2021), which we rather observe in distal leaves of EE-treated plants and not after *B. cinerea* infection. These studies demonstrate an important role of CYP71A12-dependent production of these conjugates for post-invasive resistance to the fungal pathogens *P. cucumerina*, *A. brassicicola* and *Colletotrichum tropicale*. This is a striking difference with our results showing wild-type accumulation of free ICHO/ICA as well as their conjugates in the *cyp71a12/a13* double mutant. However, *B. cinerea* can infect all the genotypes tested in our study, which is the reason why we did not discriminate between pre- and post-invasive immunity mechanisms. Despite this, it is interesting to observe these differences between pathogens with similar lifestyles and this could potentially be explained by the younger age of plants at the moment of pathogen inoculation, by the analytical methods employed or by the different pathogen strains used among studies (Bednarek et al., 2011; Pastorczyk et al., 2020). Additionally, a recent study found that germ-free *Arabidopsis* individually inoculated with a set of 39 endogenous bacteria from the phyllosphere commonly activated genes involved in indolic metabolism. The authors also showed that several indolic metabolites, including ICA, ICA-Glc and a hydroxylated form of ICA-Glc accumulated in response to bacterial inoculation. In addition, they could show that ICA and ICA-Glc accumulated significantly less in a *cyp71a12* mutant following bacterial inoculation (Maier et al., 2021). This suggests that these compounds might have a differential role in defence depending on the pathogen encountered.

Strikingly, we show that constitutive as well as EE-induced levels of ICHO/ICA conjugates are largely reduced in the *tmyb* mutant. This is in accordance with other studies which reported reduced constitutive and flg22-induced levels of ICA-Glc and 6-Glc-ICA (Frerigmann et al., 2016; Pastorczyk et al., 2020), which can be attributed to the deficiency in indolic GS accumulation or from the low constitutive expression of *CYP79B2* and *CYP79B3* genes observed in *tmyb* mutant (Frerigmann and Gigolashvili, 2014). However, we show that levels of free ICA and ICHO are not reduced in *tmyb* and still increase in response to *B. cinerea* infection, suggesting that these three MYB transcription factors might have a role in ICHO/ICA

conjugation. Interestingly, a transcriptomic analysis of MYB51-regulated genes in response to flg22 treatment identified two UGTs, *UGT85A5* and *UGT88A1*, that were down-regulated in the *myb51* single mutant (Zhou et al., 2019). We found that *UGT85A1*, a close homolog of *UGT85A5*, was significantly induced by *P. brassicae* oviposition and EE treatment but whether its induction is dependent on MYB51 under these conditions is unknown. It would be interesting to monitor the expression of the five egg-induced UGTs in the *tmyb* mutant to verify whether it correlates with the reduced accumulation of conjugates. More specifically, we observe that levels of the ICA conjugates ICA-Glc, 6-Glc-ICA-Glc and both ICHO conjugates are completely absent in *tmyb*, whereas low levels of 6-HO-ICA-Glc and 6-Glc-ICA are still detectable. It seems that direct glycosylation of ICHO and ICA is blocked in *tmyb*, but the presence of 6-Glc-ICA and 6-HO-ICA-Glc suggests another origin of the precursors with immediate glycosylation and thus escaping detection. Alternatively, formation of ICHO/ICA conjugates might derive from uncharacterised metabolites regulated by MYB34/51/122. A speculative model illustrates the above-mentioned aspects regarding the roles of MYB34/51/122 in ICHO/ICA conjugates formation (Fig. 9).

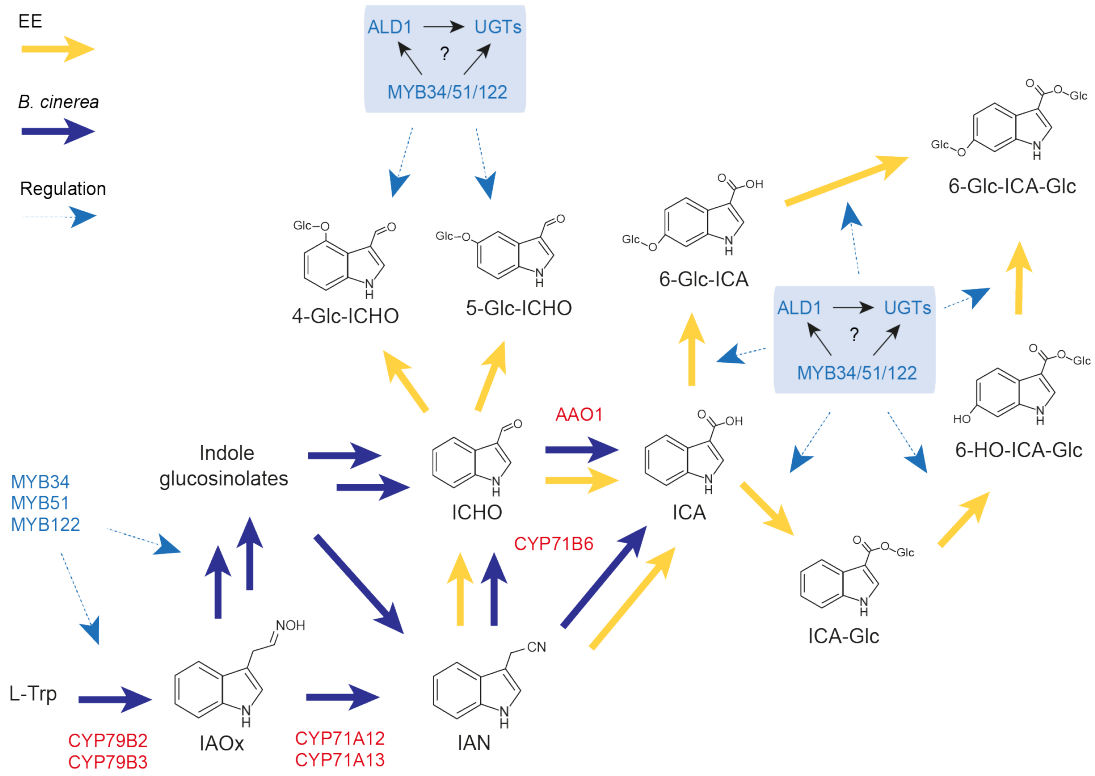


Figure 9. Model representing Trp-derived indolic metabolism and formation of ICHO/ICA conjugates.
Dark blue arrows indicate biosynthesis steps triggered following *B. cinerea* infection. Yellow arrows indicate biosynthesis steps triggered following treatment with EE for ICHO/ICA accumulation in systemic leaves. Putative interactions/regulations between ALD1, some UGTs and MYB34/51/122 are depicted in the light blue square. Refer to the text for details.

We show that ICHO/ICA conjugates accumulate systemically in response to EE treatment and that this does not correlate with EE-induced SAR establishment. Indeed, *cyp71a12/a13* accumulates significant levels of conjugates and is impaired in EE-induced SAR against *B. cinerea*, a phenomenon that we linked to the absence of camalexin in this mutant. The double *cyp71b6 aao1* mutant displays EE-induced SAR, which is attributed to the high levels of camalexin, although it also accumulates ICHO/ICA conjugates. Finally, the *tmyb* mutant is severely impacted in ICHO/ICA conjugates accumulation, which does not prevent a functional EE-induced SAR establishment against *B. cinerea*, again correlating with intact camalexin levels in this mutant. We thus conclude that systemic EE-induced ICHO and ICA conjugates accumulation is not crucial for the egg-induced SAR against *B. cinerea* but rather represents a consequence following *P. brassicae* EE treatment that could be potentially deployed for systemic protection against other pathogens.

MATERIALS AND METHODS

Plant and Insect Growth Conditions

Arabidopsis thaliana (Col-0) plants were sown in moist potting compost. After seed stratification for 2 days at 4°C, plants were grown for 4 weeks in growth chambers in short day conditions (10 h light/14 h dark), under 100 µmol m^{-2} s^{-1} of light, at 20-22°C and 65% relative humidity.

Lines used in this study: *ald1* (Návarová et al., 2012), *cyp71a12 cyp71a13* (Müller et al., 2015), *cyp71b6 aao1* (Müller et al., 2019), *cyp71b6 aao1 cyp71a12 cyp71a13* (Müller et al., 2019), *cyp79b2 cyp79b3* (Zhao et al., 2002), *ics1* (*sid2-1* allele) (Nawrath and Métraux, 1999), *myb34 myb51 myb122* (Frerigmann and Gigolashvili, 2014). All genotypes were in the Columbia (Col-0) background.

A population of the Large White butterfly *Pieris brassicae* was maintained on *Brassica oleracea* var. *gemmifera* in a greenhouse at 24°C and 65% relative humidity (Reymond et al., 2000).

Treatment with EE

For EE preparation, *P. brassicae* eggs were crushed with a pestle in Eppendorf tubes. After centrifugation (14,000 *g* for 3 min), the supernatant (EE) was collected and stored at -20°C. For application, 2 x 2 µl of EE were spotted under the surface of each of two leaves on at least 4-6 plants per independent experiment. Plants were treated 5 days before *B. cinerea* infection. Untreated plants were used as controls.

Culture of *B. cinerea* and Infection

B. cinerea strain BMM (Zimmerli et al., 2001) was grown on 1X PDA (Potato Dextrose Agar, 39 g l^{-1}, Difco) for 10-14 days in darkness at 23°C. Spores were harvested in water and filtered through wool placed in a 10 ml tip to remove hyphae. Spores were diluted in half-strength PDB (Potato Dextrose Broth, 12 g l^{-1}, Difco) to a concentration of 5 x 10^5 spores ml^{-1} for inoculation. One 5 µl droplet of spore suspension was deposited on the adaxial surface of two leaves per plant. Inoculated plants were kept under a water-sprayed transparent lid to maintain high

humidity in a growth chamber under dim light (around 2 µmol m^{-2} s^{-1}) during the whole time of infection.

Exogenous Application of Pip

One day prior to *B. cinerea* infection, 10 ml of a 1 mM D,L-Pip (Sigma-Aldrich) solution was pipetted onto each pot containing one plant. Control plants were supplemented with 10 ml of water. Each experiment was done three times (different sampling dates).

Synthesis of ICA-Glc

To a solution of ICA in dimethylformamide under nitrogen atmosphere was added cesium carbonate (Cs_2CO_3) (Sigma-Aldrich) to form ICA-Cs salts, allowing to deprotonate the acid group of ICA. Then, acetobromo-α-D-glucose tetraacetate (Sigma-Aldrich) was added for reaction with the deprotonated acid. Once formed, the intermediate acetylated ICA-glucose ester was deacetylated by adding potassium carbonate (K_2CO_3) in methanol solution. Identity of ICA-Glc (323.3 g/mol) was verified by analysis of NMR spectra. This procedure was adapted from previous reports (Southwick et al., 1986; Lorthiois et al., 2017).

Determination of Antifungal Activity

Camalexin (Glixx Laboratories, Hopkinton, USA), ICA (Sigma-Aldrich), ICHO (Sigma-Aldrich) and ICA-Glc were dissolved in dimethylsulfoxide (DMSO) before use. Round plugs with a diameter of 0.5 cm were taken from a 7-days-old *B. cinerea* culture on 1X PDA and transferred to 6-well plates supplemented with different concentration of camalexin, ICA and ICHO. Control plates contained 0.1% DMSO. For each treatment and concentration, radial growth of the fungal colony was measured on 2 plates (n=12) after 24 h of incubation at 23 °C in darkness. Mycelial growth inhibition (MGI) was calculated using the following formula: MGI % = [(C-T)/C] X 100 where C is the average colony diameter on control plates and T is the average colony diameter on treated plates. This experiment was done three times (different *B. cinerea* cultures).

Metabolite Analyses

Two leaves per plant from 5-6 plants were treated with EE for 5 days and two distal leaves were infected with *B. cinerea* spore suspension. Infected leaves were then sampled 12, 24 or 48 h later, flash-frozen in liquid nitrogen and pooled together before being ground with a mortar and pestle in nitrogen.

ICAs were profiled using a protocol adapted from Böttcher et al. (2014). An Acquity UPLC system coupled to a Synapt G2 QTOF mass spectrometer (Waters, Milford, MA) was employed. The entire system was controlled by Masslynx 4.1. The separation was performed in gradient mode on an Acquity BEH C18 column, 50x2.1mm, 1.7 µm particle size (Waters) using a flow rate of 0.4 mL/min and mobile phases consisting of H_2O + formic acid 0.05% (phase A) and acetonitrile + formic acid 0.05% (phase B). The gradient program started at 2% B, increased linearly to 60% B in 4.0 min, then to 100% B in 2.0 min, the column was then washed with 100% B for 2.0 min before re-equilibration at initial conditions (2% B) for 2.0 min. The column temperature was maintained at 25°C throughout the run. The injection volume was 2 µl (partial loop with needle overfill mode). Mass spectrometric detection was performed in electrospray negative mode using a mass range of 50-600 Da. The MS capillary voltage was -2.0 kV, the cone voltage was -25V, the desolvation temperature and gas flow were 500°C and 800 L/h, respectively, the cone gas flow was 20 L/h, and the detector voltage was 2250 V. Accurate mass measurements were provided by infusing a 500 ng/mL solution of leucine-enkephalin through the LockSpray probe at a flow rate of 15 µl/min. ICAs were identified based on their retention times and exact masses by comparison with Böttcher et al. (2014). The quantification of ICA was achieved by external calibration using calibration points at 5, 20, 100, 500 and 2000 ng/ml. All other ICAs were quantified as ICA equivalents.

Total SA was measured using the bacterial biosensor *Acinetobacter* sp. ADPWH_lux. (Huang et al., 2006; DeFraia et al., 2008), as described in Chapter 1.

For all metabolite analyses, each experiment was done at least three times (different sampling dates).

Gene Expression Analysis

To monitor gene expression, total RNA was extracted using a ReliaPrep™ RNA Tissue Miniprep System (Promega). For cDNA synthesis, 500 ng of total RNA was reverse-transcribed using M-MLV reverse transcriptase (Invitrogen) in a final volume of 15.25 µl. Each cDNA

sample was generated in triplicate and diluted eightfold with water. Quantitative real-time PCR analysis was performed in a final volume of 20 µl containing 2 µl of cDNA, 0.2 µM of each primer, 0.03 µM of reference dye and 10 µl of Brilliant III Ultra Fast SYBR Green qPCR Master Mix (Agilent). Reactions were performed using an Mx3000P real-time PCR machine (Agilent) with the following program: 95°C for 3 min, then 40 cycles of 10 sec at 95°C and 20 sec at 60°C. Relative mRNA abundance of monitored genes was normalised to the housekeeping gene *SAND* (At2g28390). Primers used are listed in the Supplemental Table 2.

Statistical Analyses

Statistical analyses were performed using R software version 3.5.2 (http://www.R-project.org). For metabolite quantifications, we used ANOVA with Tukey test for post-hoc comparison.

AUTHOR CONTRIBUTIONS

Esteban Alfonso performed the experiments and analysed data for all the figures except for Figure 2, 7 and 8 where Elia Stahl contributed to the data analyses.
Olivier Hilfiker performed the experiment for Supplemental Figure 1.
Raphaël de Matos synthesized ICA-Glc in the group of Sandrine Gerber at EPFL.
For all metabolite quantifications (Fig. 2, 3, 4B-C, 7, 8, S1, S3, S4 and S5), Esteban Alfonso treated plants and extracted samples and Gaétan Glauser processed and analysed data.
Esteban Alfonso wrote the Chapter and Philippe Reymond reviewed and edited the text.

REFERENCES

Bak S, Beisson F, Bishop G, Hamberger B, Höfer R, Paquette S, Werck-Reichhart D (2011) Cytochromes P450. The Arabidopsis Book **9**: e0144

Bednarek P (2012) Chemical warfare or modulators of defence responses – the function of secondary metabolites in plant immunity. Current Opinion in Plant Biology **15**: 407–414

Bednarek P, Pislewska-Bednarek M, Svatos A, Schneider B, Doubsky J, Mansurova M, Humphry M, Consonni C, Panstruga R, Sanchez-Vallet A, et al (2009) A Glucosinolate Metabolism Pathway in Living Plant Cells Mediates Broad-Spectrum Antifungal Defense. Science **323**: 101–106

Bednarek P, Piślewska-Bednarek M, Ver Loren van Themaat E, Maddula RK, Svatoš A, Schulze-Lefert P (2011) Conservation and clade-specific diversification of pathogen-inducible tryptophan and indole glucosinolate metabolism in Arabidopsis thaliana relatives. New Phytologist **192**: 713–726

Bednarek P, Schneider B, Svatoš A, Oldham NJ, Hahlbrock K (2005) Structural Complexity, Differential Response to Infection, and Tissue Specificity of Indolic and Phenylpropanoid Secondary Metabolism in Arabidopsis Roots. Plant Physiology **138**: 1058–1070

Bernsdorff F, Döring A-C, Gruner K, Schuck S, Bräutigam A, Zeier J (2016) Pipecolic Acid Orchestrates Plant Systemic Acquired Resistance and Defense Priming via Salicylic Acid-Dependent and -Independent Pathways. Plant Cell **28**: 102–129

Böttcher C, Chapman A, Fellermeier F, Choudhary M, Scheel D, Glawischnig E (2014) The Biosynthetic Pathway of Indole-3-Carbaldehyde and Indole-3-Carboxylic Acid Derivatives in Arabidopsis. Plant Physiology **165**: 841–853

Böttcher C, Westphal L, Schmotz C, Prade E, Scheel D, Glawischnig E (2009) The Multifunctional Enzyme CYP71B15 (PHYTOALEXIN DEFICIENT3) Converts Cysteine-Indole-3-Acetonitrile to Camalexin in the Indole-3-Acetonitrile Metabolic Network of *Arabidopsis thaliana*. The Plant Cell **21**: 1830–1845

Cecchini NM, Jung HW, Engle NL, Tschaplinski TJ, Greenberg JT (2015) ALD1 Regulates Basal Immune Components and Early Inducible Defense Responses in *Arabidopsis*. MPMI **28**: 455–466

Clay NK, Adio AM, Denoux C, Jander G, Ausubel FM (2009) Glucosinolate Metabolites Required for an Arabidopsis Innate Immune Response. Science **323**: 95–101

DeFraia CT, Schmelz EA, Mou Z (2008) A rapid biosensor-based method for quantification of free and glucose-conjugated salicylic acid. Plant Methods **4**: 28

Ding P, Rekhter D, Ding Y, Feussner K, Busta L, Haroth S, Xu S, Li X, Jetter R, Feussner I, et al (2016) Characterization of a Pipecolic Acid Biosynthesis Pathway Required for Systemic Acquired Resistance. Plant Cell **28**: 2603–2615

Ferrari S, Plotnikova JM, De Lorenzo G, Ausubel FM (2003) *Arabidopsis* local resistance to *Botrytis cinerea* involves salicylic acid and camalexin and requires *EDS4* and *PAD2* , but not *SID2* , *EDS5* or *PAD4*: *Local* Arabidopsis *response to* Botrytis. The Plant Journal **35**: 193–205

Forcat S, Bennett M, Grant M, Mansfield JW (2010) Rapid linkage of indole carboxylic acid to the plant cell wall identified as a component of basal defence in Arabidopsis against hrp mutant bacteria. Phytochemistry **71**: 870–876

Frerigmann H, Gigolashvili T (2014) MYB34, MYB51, and MYB122 Distinctly Regulate Indolic Glucosinolate Biosynthesis in Arabidopsis thaliana. Molecular Plant **7**: 814–828

Frerigmann H, Piślewska-Bednarek M, Sánchez-Vallet A, Molina A, Glawischnig E, Gigolashvili T, Bednarek P (2016) Regulation of Pathogen-Triggered Tryptophan Metabolism in Arabidopsis thaliana by MYB Transcription Factors and Indole Glucosinolate Conversion Products. Molecular Plant **9**: 682–695

Gachon CMM, Langlois-Meurinne M, Saindrenan P (2005) Plant secondary metabolism glycosyltransferases: the emerging functional analysis. Trends in Plant Science **10**: 542–549

Gamir J, Pastor V, Cerezo M, Flors V (2012) Identification of indole-3-carboxylic acid as mediator of priming against Plectosphaerella cucumerina. Plant Physiology and Biochemistry **61**: 169–179

Gamir J, Pastor V, Kaever A, Cerezo M, Flors V (2014) Targeting novel chemical and constitutive primed metabolites against *Plectosphaerella cucumerina*. Plant J **78**: 227–240

Glawischnig E (2007) Camalexin. Phytochemistry **68**: 401–406

Gouhier-Darimont C, Schmiesing A, Bonnet C, Lassueur S, Reymond P (2013) Signalling of Arabidopsis thaliana response to Pieris brassicae eggs shares similarities with PAMP-triggered immunity. Journal of Experimental Botany **64**: 665–674

Hagemeier J, Schneider B, Oldham NJ, Hahlbrock K (2001) Accumulation of soluble and wall-bound indolic metabolites in Arabidopsis thaliana leaves infected with virulent or avirulent Pseudomonas syringae pathovar tomato strains. Proceedings of the National Academy of Sciences **98**: 753–758

Hartmann M, Kim D, Bernsdorff F, Ajami-Rashidi Z, Scholten N, Schreiber S, Zeier T, Schuck S, Reichel-Deland V, Zeier J (2017) Biochemical Principles and Functional Aspects of Pipecolic Acid Biosynthesis in Plant Immunity. Plant Physiol **174**: 124–153

Hartmann M, Zeier J (2019) N-hydroxypipecolic acid and salicylic acid: a metabolic duo for systemic acquired resistance. Current Opinion in Plant Biology **50**: 44–57

Hilfiker O, Groux R, Bruessow F, Kiefer K, Zeier J, Reymond P (2014) Insect eggs induce a systemic acquired resistance in Arabidopsis. Plant J **80**: 1085–1094

Hopkins RJ, van Dam NM, van Loon JJA (2009) Role of Glucosinolates in Insect-Plant Relationships and Multitrophic Interactions. Annu Rev Entomol **54**: 57–83

Huang WE, Huang L, Preston GM, Naylor M, Carr JP, Li Y, Singer AC, Whiteley AS, Wang H (2006) Quantitative *in situ* assay of salicylic acid in tobacco leaves using a genetically modified biosensor strain of *Acinetobacter* sp. ADP1. The Plant Journal **46**: 1073–1083

Jackson RG, Lim E-K, Li Y, Kowalczyk M, Sandberg G, Hoggett J, Ashford DA, Bowles DJ (2001) Identification and Biochemical Characterization of anArabidopsis Indole-3-acetic Acid Glucosyltransferase. Journal of Biological Chemistry **276**: 4350–4356

Jin S-H, Ma X-M, Han P, Wang B, Sun Y-G, Zhang G-Z, Li Y-J, Hou B-K (2013) UGT74D1 Is a Novel Auxin Glycosyltransferase from Arabidopsis thaliana. PLoS ONE **8**: e61705

Kettles GJ, Drurey C, Schoonbeek H, Maule AJ, Hogenhout SA (2013) Resistance of *A rabidopsis thaliana* to the green peach aphid, *M yzus persicae* , involves camalexin and is regulated by micro RNA s. New Phytol **198**: 1178–1190

Kim JH, Lee BW, Schroeder FC, Jander G (2008) Identification of indole glucosinolate breakdown products with antifeedant effects on Myzus persicae (green peach aphid). Plant J **54**: 1015–1026

Klein AP, Anarat-Cappillino G, Sattely ES (2013) Minimum Set of Cytochromes P450 for Reconstituting the Biosynthesis of Camalexin, a Major *Arabidopsis* Antibiotic. Angew Chem Int Ed **52**: 13625–13628

Kliebenstein DJ, Rowe HC, Denby KJ (2005) Secondary metabolites influence Arabidopsis/Botrytis interactions: variation in host production and pathogen sensitivity: Secondary metabolites influence Arabidopsis/Botrytis interactions. The Plant Journal **44**: 25–36

Koiwai H, Akaba S, Seo M, Komano T, Koshiba T (2000) Functional Expression of Two Arabidopsis Aldehyde Oxidases in the Yeast Pichia pastoris. Journal of Biochemistry **127**: 659–664

Kosaka A, Pastorczyk M, Piślewska-Bednarek M, Nishiuchi T, Ono E, Suemoto H, Ishikawa A, Frerigmann H, Kaido M, Mise K, et al (2021) Tryptophan-derived metabolites and BAK1 separately contribute to Arabidopsis postinvasive immunity against Alternaria brassicicola. Sci Rep **11**: 1488

Langlois-Meurinne M, Gachon CMM, Saindrenan P (2005) Pathogen-Responsive Expression of Glycosyltransferase Genes *UGT73B3* and *UGT73B5* Is Necessary for Resistance to *Pseudomonas syringae* pv *tomato* in Arabidopsis. Plant Physiology **139**: 1890–1901

Li Y, Baldauf S, Lim E-K, Bowles DJ (2001) Phylogenetic Analysis of the UDP-glycosyltransferase Multigene Family of Arabidopsis thaliana. Journal of Biological Chemistry **276**: 4338–4343

Lorthiois E, Anderson K, Vulpetti A, Rogel O, Cumin F, Ostermann N, Steinbacher S, Mac Sweeney A, Delgado O, Liao S-M, et al (2017) Discovery of Highly Potent and Selective Small-Molecule Reversible Factor D Inhibitors Demonstrating Alternative Complement Pathway Inhibition *in Vivo*. J Med Chem **60**: 5717–5735

Maier BA, Kiefer P, Field CM, Hemmerle L, Bortfield-Miller M, Emmenegger B, Schäfer M, Pfellmeier S, Sunagawa S, Vogel CM, et al (2021) A general non-self response as part of plant immunity. Nature Plants **7**: 28

Mikkelsen MD, Hansen CH, Wittstock U, Halkier BA (2000) Cytochrome P450 CYP79B2 from Arabidopsis Catalyzes the Conversion of Tryptophan to Indole-3-acetaldoxime, a Precursor of Indole Glucosinolates and Indole-3-acetic Acid. Journal of Biological Chemistry **275**: 33712–33717

Millet YA, Danna CH, Clay NK, Songnuan W, Simon MD, Werck-Reichhart D, Ausubel FM (2010) Innate Immune Responses Activated in *Arabidopsis* Roots by Microbe-Associated Molecular Patterns. The Plant Cell **22**: 973–990

Mucha S, Heinzlmeir S, Kriechbaumer V, Strickland B, Kirchhelle C, Choudhary M, Kowalski N, Eichmann R, Hueckelhoven R, Grill E, et al (2019) The formation of a camalexin-biosynthetic metabolon. Plant Cell tpc.00403.2019

Müller TM, Böttcher C, Glawischnig E (2019) Dissection of the network of indolic defence compounds in Arabidopsis thaliana by multiple mutant analysis. Phytochemistry **161**: 11–20

Müller TM, Böttcher C, Morbitzer R, Götz CC, Lehmann J, Lahaye T, Glawischnig E (2015) TRANSCRIPTION ACTIVATOR-LIKE EFFECTOR NUCLEASE-Mediated Generation and Metabolic Analysis of Camalexin-Deficient *cyp71a12 cyp71a13* Double Knockout Lines. Plant Physiology **168**: 849–858

Nafisi M, Goregaoker S, Botanga CJ, Glawischnig E, Olsen CE, Halkier BA, Glazebrook J (2007) *Arabidopsis* Cytochrome P450 Monooxygenase 71A13 Catalyzes the Conversion of Indole-3-Acetaldoxime in Camalexin Synthesis. The Plant Cell **19**: 2039–2052

Návarová H, Bernsdorff F, Döring A-C, Zeier J (2012) Pipecolic Acid, an Endogenous Mediator of Defense Amplification and Priming, Is a Critical Regulator of Inducible Plant Immunity. Plant Cell **24**: 5123–5141

Nawrath C, Métraux J-P (1999) Salicylic Acid Induction–Deficient Mutants of Arabidopsis Express PR-2 and PR-5 and Accumulate High Levels of Camalexin after Pathogen Inoculation. 13

Oka T, Jigami Y (2006) Reconstruction of *de novo* pathway for synthesis of UDP-glucuronic acid and UDP-xylose from intrinsic UDP-glucose in *Saccharomyces cerevisiae*. FEBS Journal **273**: 2645–2657

Pastorczyk M, Kosaka A, Piślewska-Bednarek M, López G, Frerigmann H, Kułak K, Glawischnig E, Molina A, Takano Y, Bednarek P (2020) The role of CYP 71A12 monooxygenase in pathogen-triggered tryptophan metabolism and Arabidopsis immunity. New Phytol **225**: 400–412

Reymond P, Weber H, Damond M, Farmer EE (2000) Differential Gene Expression in Response to Mechanical Wounding and Insect Feeding in Arabidopsis. 14

Schuhegger R, Nafisi M, Mansourova M, Petersen BL, Olsen CE, Svatoš A, Halkier BA, Glawischnig E (2006) CYP71B15 (PAD3) Catalyzes the Final Step in Camalexin Biosynthesis. Plant Physiology **141**: 1248–1254

Song JT, Lu H, Greenberg JT (2004a) Divergent Roles in *Arabidopsis thaliana* Development and Defense of Two Homologous Genes, *ABERRANT GROWTH AND DEATH2* and *AGD2-LIKE DEFENSE RESPONSE PROTEIN1* , Encoding Novel Aminotransferases. Plant Cell **16**: 353–366

Song JT, Lu H, McDowell JM, Greenberg JT (2004b) A key role for ALD1 in activation of local and systemic defenses in Arabidopsis: A key role for ALD1 in defense signaling. The Plant Journal **40**: 200–212

Southwick SM, Chung A, Davenport TL, Ryan JW (1986) A Rapid, Simple Synthesis and Purification of Abscisic Acid Glucose Ester. Plant Physiol **81**: 323–325

Stahl E, Bellwon P, Huber S, Schlaeppi K, Bernsdorff F, Vallat-Michel A, Mauch F, Zeier J (2016) Regulatory and Functional Aspects of Indolic Metabolism in Plant Systemic Acquired Resistance. Molecular Plant **9**: 662–681

Stahl E, Brillatz T, Ferreira Queiroz E, Marcourt L, Schmiesing A, Hilfiker O, Riezman I, Riezman H, Wolfender J-L, Reymond P (2020) Phosphatidylcholines from Pieris brassicae eggs activate an immune response in Arabidopsis. eLife **9**: e60293

Tan J, Bednarek P, Liu J, Schneider B, Svatoš A, Hahlbrock K (2004) Universally occurring phenylpropanoid and species-specific indolic metabolites in infected and uninfected Arabidopsis thaliana roots and leaves. Phytochemistry **65**: 691–699

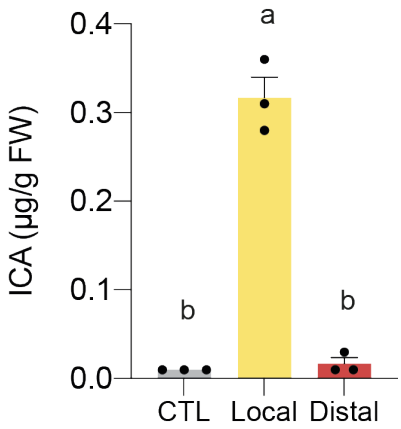
de Vos M, Kriksunov KL, Jander G (2008) Indole-3-Acetonitrile Production from Indole Glucosinolates Deters Oviposition by *Pieris rapae*. Plant Physiol **146**: 916–926

Zhao Y, Hull AK, Gupta NR, Goss KA, Alonso J, Ecker JR, Normanly J, Chory J, Celenza JL (2002) Trp-dependent auxin biosynthesis in Arabidopsis: involvement of cytochrome P450s CYP79B2 and CYP79B3. 13

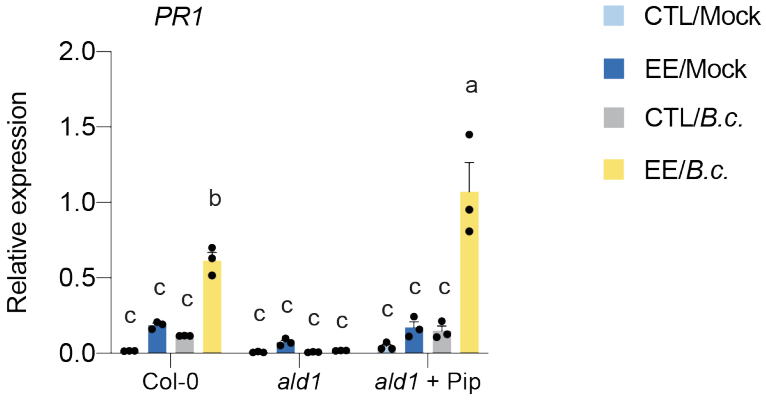
Zhou J, Kong W, Zhao H, Li R, Yang Y, Li J (2019) Transcriptome-wide identification of indole glucosinolate dependent flg22-response genes in Arabidopsis. Biochemical and Biophysical Research Communications **520**: 311–319

Zimmerli L, Métraux J-P, Mauch-Mani B (2001) β-Aminobutyric Acid-Induced Protection of Arabidopsis against the Necrotrophic Fungus Botrytis cinerea. Plant Physiology **126**: 517–523

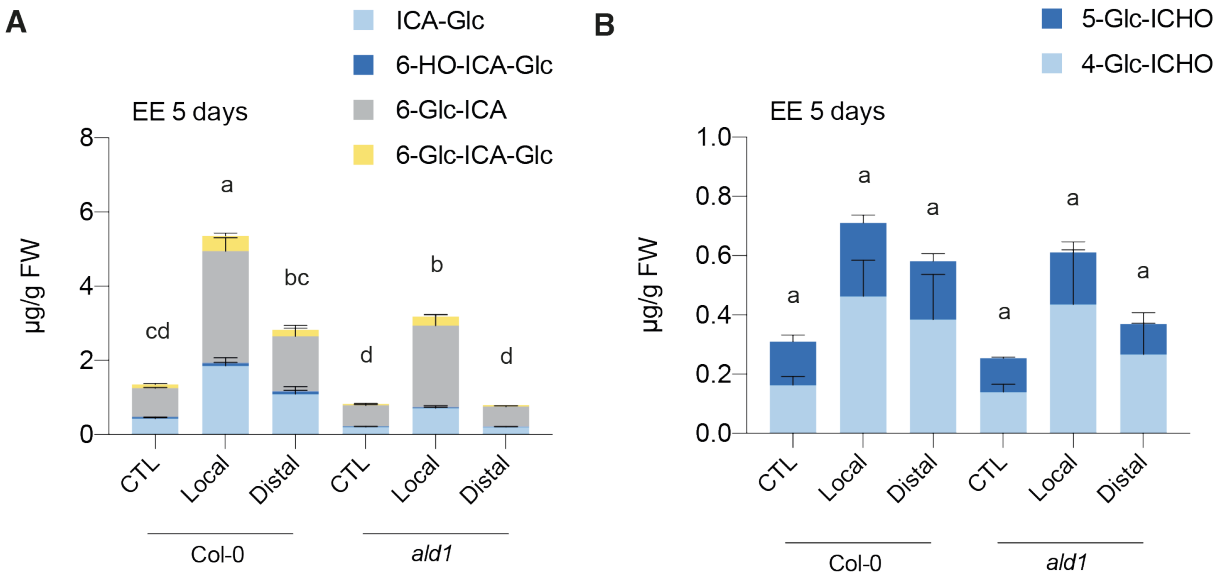
SUPPLEMENTAL DATA



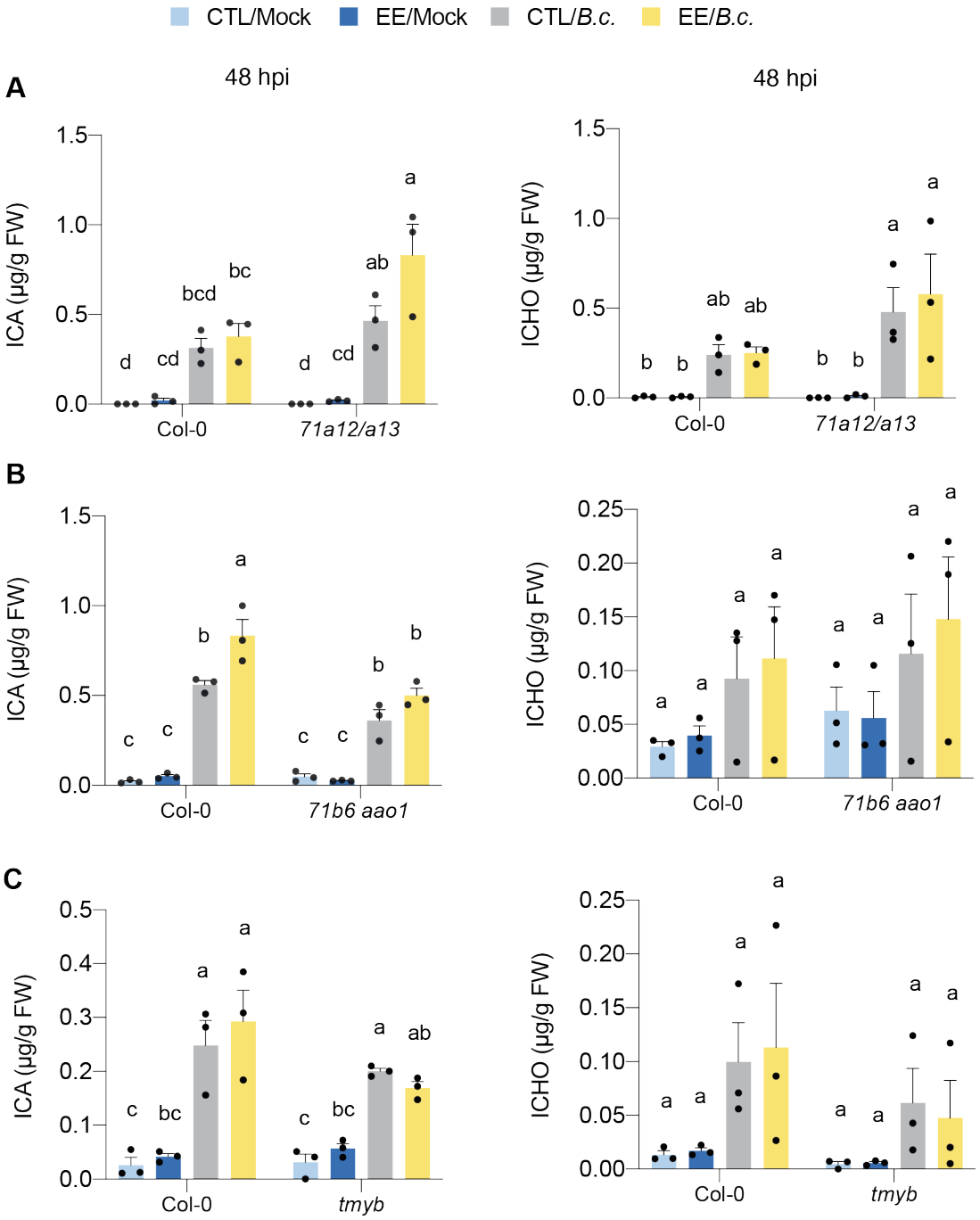
Supplemental Figure 1. ICA accumulates in EE-treated leaves.
Col-0 plants were treated with *P. brassicae* EE for 5 days or left untreated. ICA levels were measured in untreated leaves (CTL), EE-treated leaves (Local) and leaves systemic from EE-treated leaves (Distal). Means ± SE of three independent experiments are shown (n = 10-12 leaves per experiment). Different letters indicate significant difference at $P < 0.05$ (ANOVA followed by Tukey's Honest Significant Difference test). Dots indicate individual values.



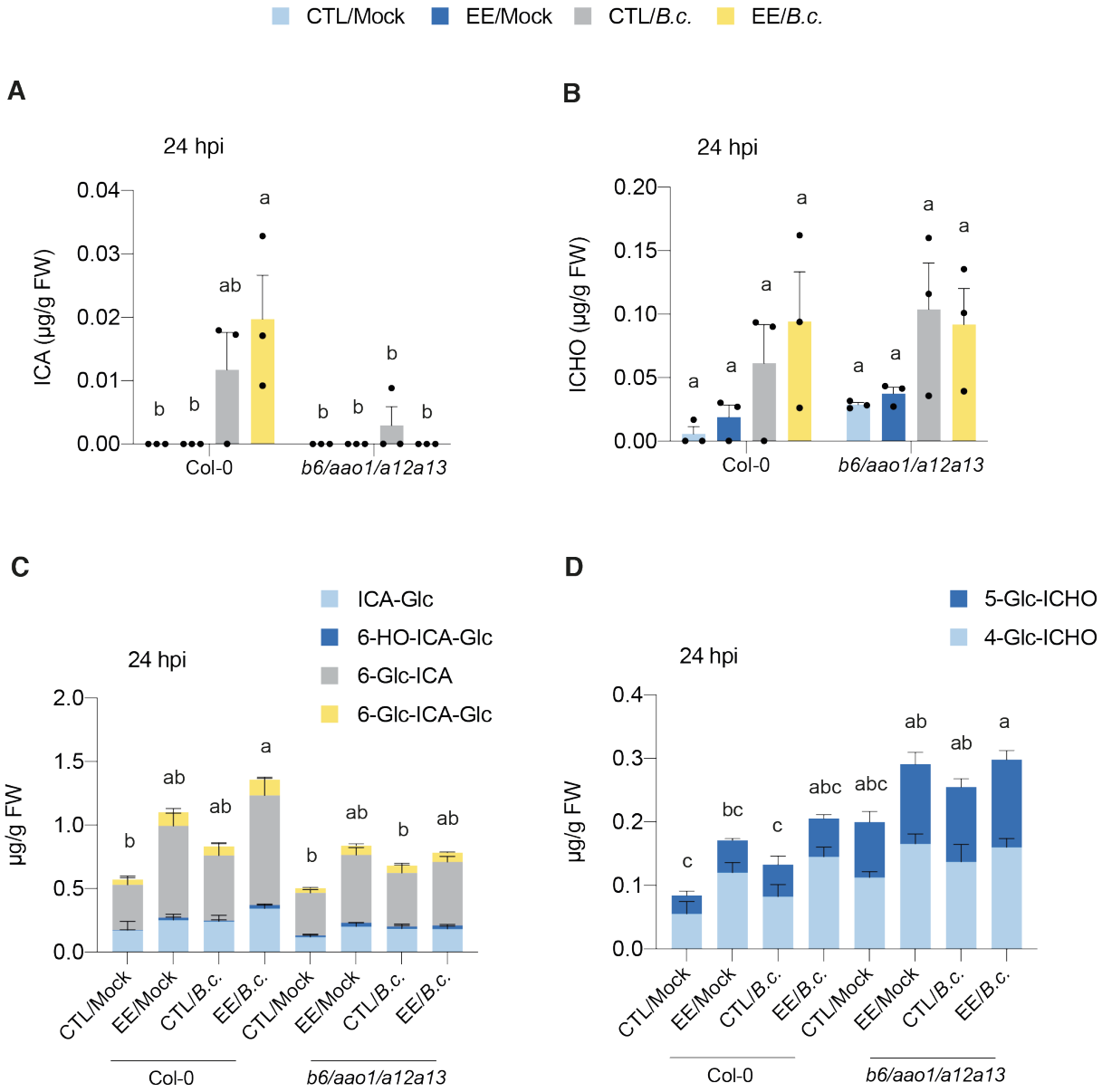
Supplemental Figure 2. Pip watering restores EE-induced priming of *PR1* expression in *ald1*.
Local leaves were left untreated (CTL) or pretreated with *P. brassicae* EE for 5 days (EE) and distal leaves were further inoculated with *B. cinerea* spore suspension (*B.c.*) or a mock solution (Mock) for 24 h. Water or 1 mM pipecolic acid (Pip) was applied to the soil one day prior inoculation. Expression of *PR1* gene was monitored 24 hpi. Means ± SE of three technical replicates of one experiment are shown (n = 10-12 leaves). The experiment was repeated once with similar results. Different letters indicate significant difference at $P < 0.05$ (ANOVA followed by Tukey's Honest Significant Difference test). Dots indicate individual values.



Supplemental Figure 3. ICHO/ICA conjugates accumulation in *ald1* following EE treatment.
Plant genotypes were treated with *P. brassicae* EE for 5 days. ICA (**A**) and ICHO (**B**) conjugates were measured in untreated leaves (CTL), EE-treated leaves (Local) and leaves distal from EE-treated leaves (Distal). Means ± SE of three independent experiments are shown (n = 10-12 leaves per experiment). Different letters indicate significant difference at $P< 0.05$ (ANOVA followed by Tukey's Honest Significant Difference test).



Supplemental Figure 4. ICA and ICHO accumulation in indolic mutants.
Local leaves were left untreated (CTL) or pretreated with *P. brassicae* EE for 5 days (EE) and distal leaves were further inoculated with *B. cinerea* spore suspension (*B.c.*) or a mock solution (Mock) for 24 h and 48 h. ICA and ICHO levels were measured in distal leaves of *cyp71a12 cyp71a13* (*71a12/a13*) double mutant (**A**), *cyp71b6 aao1* (*71b6 aao1*) double mutant (**B**) and *myb34 myb51 myb122* triple mutant (*tmyb*) (**C**). Means ± SE of three independent experiments are shown (n = 10-12 leaves per experiment). Different letters indicate significant difference at $P < 0.05$ (ANOVA followed by Tukey's Honest Significant Difference test). Dots indicate individual values.



Supplemental Figure 5. ICA, ICHO and their conjugates accumulate in *cyp71b6 aao1 cyp71a12 cyp71a13* quadruple mutant.
Local leaves were left untreated (CTL) or pretreated with *P. brassicae* EE for 5 days (EE) and distal leaves were further inoculated with *B. cinerea* spore suspension (*B.c.*) or a mock solution (Mock) for 24 h. ICA (**A**), ICHO (**B**), ICA conjugates (**C**) and ICHO conjugates (**D**) levels were measured in distal leaves. Means ± SE of three independent experiments are shown (n = 10-12 leaves per experiment). Different letters indicate significant difference at $P < 0.05$ (ANOVA followed by Tukey's Honest Significant Difference test). Dots indicate individual values.

Supplemental Table 1. List of UGT genes induced in local leaves following 5 days of *P. brassicae* oviposition or treatment with EE. These data were extracted from RNA sequencing experiments published in Stahl et al. (2020).

Oviposition Local 5d				EE Local 5d				Ovi + EE Local 5d	
AGI Code	Gene Name	logFC	adj.P.Val	AGI Code	Gene Name	logFC	adj.P.Val	AGI Code	Gene Name
AT3G46700	UGT76E3	5,656	0,00059	AT2G15490	UGT73B4	8,265	0,00024	AT3G46700	UGT76E3
AT3G53150	UGT73D1	5,387	0,00137	AT3G46690	UGT76E4	4,454	0,00038	AT3G53150	UGT73D1
AT3G10320	MUCI21	5,269	0,00111	AT1G22340	UGT85A7	2,419	0,00067	AT3G10320	MUCI21
AT2G36780	UGT73C3	5,760	0,00231	AT3G53150	UGT73D1	5,808	0,00081	AT3G46690	UGT76E4
AT3G46690	UGT76E4	3,240	0,00360	AT4G34131	UGT73B3	4,077	0,00081	AT3G46660	UGT76E12
AT3G46660	UGT76E12	5,671	0,00384	AT4G15280	UGT71B5	6,629	0,00118	AT4G34131	UGT73B3
AT4G34131	UGT73B3	3,226	0,00404	AT3G46700	UGT76E3	5,038	0,00133	AT1G22340	UGT85A7
AT1G22340	UGT85A7	1,829	0,00455	AT3G46660	UGT76E12	6,549	0,00145	AT2G26480	UGT76D1
AT2G26480	UGT76D1	4,122	0,00796	AT1G07260	UGT71C3	3,949	0,00213	AT2G30140	UGT87A2
AT2G30140	UGT87A2	1,623	0,00850	AT2G36780	UGT73C3	5,817	0,00217	AT4G15280	UGT71B5
AT4G15280	UGT71B5	4,770	0,01040	AT2G30140	UGT87A2	1,862	0,00351	AT4G34135	UGT73B2
AT2G36750	UGT73C1	3,533	0,01835	AT3G53160	UGT73C7	1,270	0,00352	AT1G22400	UGT85A1
AT4G34135	UGT73B2	1,653	0,02273	AT1G22400	UGT85A1	3,221	0,01291	AT3G57380	F28O9.230
AT1G22400	UGT85A1	2,808	0,02808	AT2G15480	UGT73B5	1,543	0,01328	AT4G15270	DL3680C
AT3G57380	F28O9.230	3,550	0,00749	AT4G34135	UGT73B2	1,813	0,01341	AT2G36800	UGT73C5
AT4G15270	DL3680C	4,759	0,00153	AT3G21790	UGT71B7	1,408	0,02406	AT2G36780	UGT73C3
AT2G36800	UGT73C5	1,580	0,02616	AT2G36970	UGT86A1	1,798	0,02508		
				AT3G50740	UGT72E1	1,324	0,02915		
				AT2G26480	UGT76D1	3,229	0,03243		
				AT3G21780	UGT71B6	1,995	0,04543		
				AT3G10320	F14P13.8	6,148	0,00036		
				AT3G57380	F28O9.230	4,031	0,00327		
				AT3G09020	T16O11.2	1,445	0,00380		
				AT4G15270	DL3680C	7,286	0,00006		
				AT2G36800	UGT73C5	1,493	0,03534		

Supplemental Table 2. List of primers used for RT-qPCR.

Gene name	Gene ID	Primers ID	Sequences (5'-3')	Reference
SAND	At2g28390	SAND-Fw	AACTCTATGCAGCATTTGATCCACT	Gouhier-Darimont et al. (2013)
		SAND-Rv	TGATTGCATATCTTTATCGCCATC	
PR1	At2g14610	PR1-Fw	GTGGGTTAGCGAGAAGGCTA	Gouhier-Darimont et al. (2013)
		PR1-Rv	ACTTTGGCACATCCGAGTCT	
UGT73D1	At3g53150	UGT73D1-Fw	AAGAAACCGAGTGTTGTGAAAGC	Langlois-Meurinne et al. (2005)
		UGT73D1-Rv	TCATTATCATCATCATTTTCGTCTACAC	
UGT73B2	At4g34135	UGT73B2-Fw	AGTTAAATTCAAATGGCAGCAACC	Langlois-Meurinne et al. (2005)
		UGT73B2-Rv	TCTTGAACCATTGATTTTCTCCTAAC	
UGT73B3	At4g34131	UGT73B3-Fw	ATAGCTTCATTGAAAAGACCTCAGTAAG	Langlois-Meurinne et al. (2005)
		UGT73B3-Rv	CCAAGACAAAGACTAAGCAGAATCG	
UGT76D1	At2g26480	UGT76D1-Fw	CAACTGCACAAGAGAAATGGGG	
		UGT76D1-Rv	AAACTTGGACATGACATTGCGG	
UGT85A1	At1g22400	UGT85A1-Fw	TCCGATTTGAGTCCATTGCTGA	
		UGT85A1-Rv	CGAGACAGTTCTTCATGGTGGA	

CHAPTER 4

Biological relevance and generality of egg-induced systemic acquired resistance

Esteban Alfonso[1], Etienne Bellani[1], Tom M. Raaymakers[2], Guido Van den Ackerveken[2] and Philippe Reymond[1]

[1]Department of Plant Molecular Biology, University of Lausanne, Switzerland
[2]Plant-Microbe Interactions, Department of Biology, Utrecht University, The Netherlands

ABSTRACT

In *Arabidopsis*, insect eggs deposition activates plant defences and triggers the accumulation of salicylic acid (SA). Egg-induced SA accumulation is required to induce systemic protection against bacterial and fungal pathogens, a process termed systemic acquired resistance (SAR). Moreover, egg-induced SAR against the bacterial pathogen *Pseudomonas syringae* also extends to egg-free neighbouring plants through a root-mediated signal. Here, we address the biological relevance of EE-induced SAR against the necrotroph *Botrytis cinerea*. We show that *Pieris brassicae* larvae performance is reduced on *B. cinerea*-infected plants, implying a beneficial role of EE-induced SAR for the insect. We demonstrate that EE-induced SAR against *B. cinerea* also extends to neighbouring plants. In addition, we show that treatment with EE induces local protection towards *B. cinerea* in other *Brassicaceae* plant species. Finally, EE-induced SAR is also effective against *Hyaloperonospora arabidopsidis*, broadening the protective effect of insect eggs to an oomycete.

INTRODUCTION

In nature, plants interact simultaneously with insects and pathogens, each of them triggering similar and/or contrasting signalling pathways. Such tripartite interactions can lead to various outcomes for the host plant and the attackers, depending on the environment, host metabolism and interplay of signalling pathways (Stout et al., 2006). Oviposition by the specialist herbivore *Pieris brassicae* triggers the activation of the salicylic acid (SA) pathway in *Arabidopsis* (Little et al., 2007; Bruessow et al., 2010), which is usually produced to fend off biotrophic pathogens. In *Arabidopsis*, *P. brassicae* oviposition and egg extract (EE) treatment induce SAR in *Arabidopsis* against the hemibiotroph *Pseudomonas syringae* (Hilfiker et al., 2014). SAR depends on the SA pathway and requires the translocation of a SAR signal to efficiently enhance defence responses in systemic tissues (Fu and Dong, 2013). Several SAR signal candidates have been described, including the pipecolic acid (Pip)-derivative *N*-hydroxy-Pip (NHP), which has been shown to efficiently coordinate SAR along with SA (Hartmann and Zeier, 2019). The first step of NHP biosynthesis is catalysed by ALD1 to generate Pip and the *ald1* mutant is compromised in bacterial- and egg-induced SAR (Návarová et al., 2012; Hilfiker et al., 2014). Pip is then further *N*-hydroxylated by FMO1, generating NHP, the actual SAR regulator (Chen et al., 2018; Hartmann et al., 2018).

Upon perception of stress, plants release various above- and below-ground signals, including volatiles organic compounds (VOCs) and small metabolites. These signals can be perceived by neighbouring plants and serve as cues for imminent danger (Delory et al., 2016; Ninkovic et al., 2019). SAR-induced plants have been shown to emit volatile cues, which can induce SAR in neighbouring plants (Riedlmeier et al., 2017; Wenig et al., 2019). Generation of below-ground SAR signal has also been shown to enhance protection against pathogens in neighbouring plants (Cheol Song et al., 2016). Recently, it was shown that *P. brassicae* egg-induced SAR against *P. syringae* extends to neighbouring plants (interplant SAR), through the transmission of a root-mediated signal generated in a NHP-dependent manner (Orlovskis and Reymond, 2020).

Oviposition-induced responses, such as intra- and interplant SAR raise the question of whether the plant or the insect benefits from reduced foliar pathogen load. It was previously shown that egg-induced SA accumulation can locally inhibit induction of the jasmonic acid (JA) pathway, leading to increased performance of the generalist herbivore *Spodoptera littoralis* (Bruessow et al., 2010). In addition, performance of *P. brassicae* larvae is decreased when fed on *P. syringae*-infected plants, and this was partially rescued when fed on plants

previously treated with EE (Hilfiker et al., 2014), which suggests that egg-induced SAR might benefit the insect.

SAR against fungal pathogens has been shown to establish in field conditions, in plants of agricultural importance, such as bean, cucumber or rice (Sticher et al., 1997). However, the SAR-inducing stimulus was either a primary infection by a bacterial or fungal pathogen, or treatment with synthetic elicitors. Given that insect oviposition induces SAR in *Arabidopsis* against pathogens with different lifestyles, chances are great that it might also activate local and systemic resistance in other plants. Recently, it was shown that various monocotyledonous and dicotyledonous species infected with adapted pathogens accumulate NHP and that exogenous treatment with this metabolite induces acquired resistance (Schnake et al., 2020), indicating that this pathway is widespread in plants.

In this chapter, we investigate the biological relevance and generality of EE-induced SAR against *Botrytis cinerea*. We show that EE-induced SAR extends its protection towards *B. cinerea* in egg-free neighbouring plants. We also show that *B. cinerea* infection is reduced in local EE-treated leaves of other plants from the *Brassicaceae* family, but not in tomato. Moreover, *P. brassicae* larval performance is reduced in *B. cinerea*-infected *Arabidopsis*. Finally, we also demonstrate that EE-induced SAR is effective against the oomycete *Hyaloperonospora arabidopsidis*, indicating that oviposition protects plants against a broad range of pathogens.

RESULTS

Treatment with *Pieris brassicae* egg extract induces interplant SAR

It was shown recently that egg-induced SAR against *P. syringae* extends to egg-free neighbouring plants through an unknown root-mediated signal (Orlovskis and Reymond, 2020). We thus investigated whether egg-induced interplant SAR was also efficient against *B. cinerea*. For that, four *Arabidopsis* plants were grown in the same pot. Two plants out of four were treated with *P. brassicae* EE for five days. We then infected distal leaves of EE-treated plants as well as leaves of two untreated neighbouring plants with *B. cinerea*. We found that *B. cinerea* growth was significantly reduced in neighbouring plants, similar to distal leaves of EE-treated plants, compared to untreated control plants (Fig. 1), indicating that EE-induced interplant SAR is also effective against *B. cinerea*.

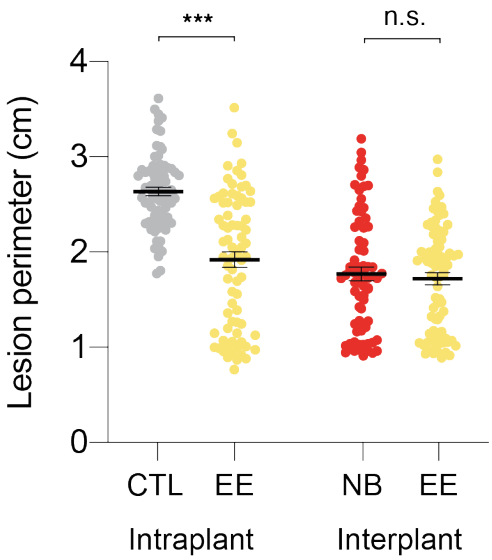


Figure 1. Treatment with *Pieris brassicae* EE induces interplant SAR against *Botrytis cinerea*.
EE pretreatment reduced *B. cinerea* growth in distal leaves when compared to CTL plants grown separately (intraplant SAR). No difference in *B. cinerea* growth was observed when EE-treated and untreated neighbor plants (NB) were in the same pot (interplant SAR). Means ± SE of three independent experiments are shown (n = 22-30 leaves per experiment). Significant differences between control and treated plants are indicated (linear mixed model, *** P<0.001, n.s. not significant). Dots indicate individual values.

P. brassicae EE treatment reduces *B. cinerea* growth in other *Brassicaceae* species

B. cinerea is a highly polyphagous fungal pathogen, able to infect more than 1000 plant species, including several important crops (Williamson et al., 2007; Dean et al., 2012). As *P. brassicae* oviposition and EE treatment reduce *B. cinerea* growth in *Arabidopsis*, we investigated whether this could be observed in other plant species. We thus selected several *Brassicaceae* crops for bioassays, including rapeseed (*Brassica napus*), Brussels sprouts (*Brassica oleracea*), black mustard (*Brassica nigra*) and pink radish (*Raphanus sativus*). As previously described, two leaves per plant were treated with *P. brassicae* EE for 5 days and two distal leaves were subsequently infected with *B. cinerea*. In addition, we also infected local EE-treated leaves. Strikingly, we found that EE pretreatment induced a reduction of *B. cinerea* growth in local leaves of all four tested species (Fig. 2). However, EE-induced fungal growth reduction in distal leaves only occurred in *B. napus*, with no significant difference compared to untreated control plants for the other species (Fig. 2). In addition, we also pretreated with EE two leaves of tomato (*Solanum lycopersicum* accession Micro-Tom) and subsequently infected local and distal leaves. Interestingly, EE pretreatment did not induce *B. cinerea* growth reduction in both local and distal leaves (Fig. 2).

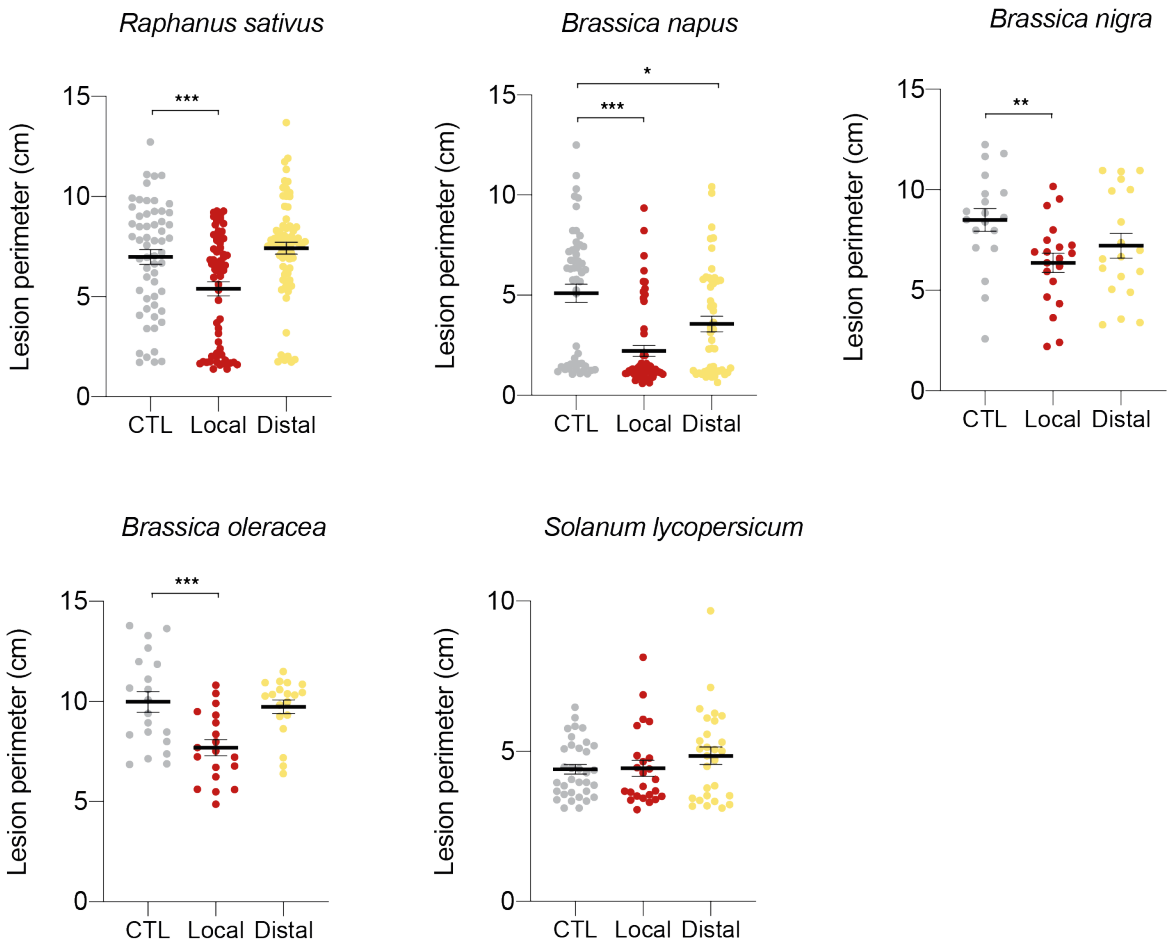


Figure 2. EE treatment reduces *B. cinerea* growth in other plant species.
Two leaves per plant were pretreated with *P. brassicae* EE for 5 days and two distal leaves were further infected with *B. cinerea* for 5 days (*R. sativus*, *B. napus* and *S. lycopersicum*) or 7 days (*B. nigra* and *B. oleracea*). Lesion perimeter was measured in control (CTL), EE-treated leaves (Local) and in leaves distal from EE-treated leaves (Distal). Means ± SE of three independent experiments are shown (n = 5-30 leaves per experiment). Significant differences between CTL and treatment are indicated (linear mixed model, *** P<0.001; ** P<0.01; * P<0.05). Dots indicate individual values.

B. cinerea infection impacts *P. brassicae* larval performance

Primary infection of plants with pathogens can either negatively or positively impact herbivore performance (Stout et al., 2006). We thus decided to investigate the biological relevance of EE-induced SAR to see whether it might benefit future hatching larvae. We measured the performance of *P. brassicae* larvae on *B. cinerea*-infected plants. *Arabidopsis* plants were sprayed with a suspension of *B. cinerea* spores and after two days of infection, newly hatched *P. brassicae* larvae were placed on infected plants and mock-inoculated control plants. Larval weight was measured after 6 and 12 days. After 12 days, *P. brassicae* larvae were significantly smaller when feeding on infected plants compared to plants sprayed with the control mock

solution (Fig. 3). This suggests that EE-induced SAR against *B. cinerea* might benefit hatching larvae.

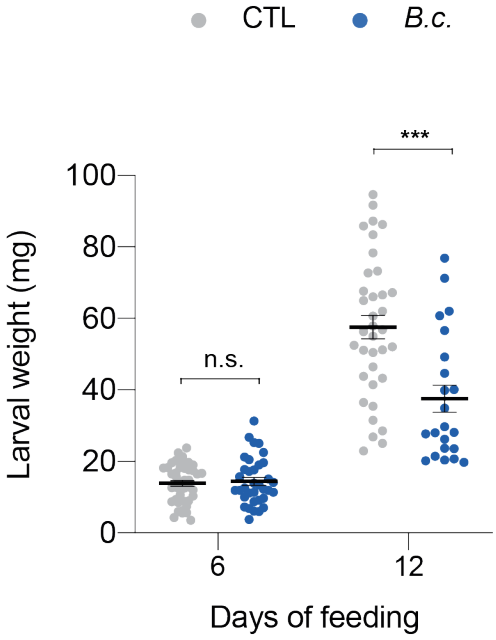


Figure 3. *P. brassicae* larval development is inhibited in *B. cinerea*-infected plants.
Plants were sprayed with a suspension of *B. cinerea* spores (*B.c.*) or mock solution (CTL). Freshly hatched *P. brassicae* were then placed on plants for a total of 12 days. Newly infected plants were placed every 3 days, in order to have sufficient material for the larvae to feed on. Larval weight was recorded after 6 and 12 days. Means ± SE are shown (n = 22-43). Significant differences between control and infected plants are indicated (Welch's two sample *t*-test, *** P<0.001; n.s., not significant). This experiment was repeated twice with similar results. Dots indicate individual values.

EE-induced SAR against *Hyaloperonospora arabidopsidis*

To test whether EE-induced SAR can target other plant pathogens, we monitored infection of the oomycete *H. arabidopsidis* (*Hpa*), which is an obligate biotroph that causes downy mildew on *Arabidopsis* (Coates and Beynon, 2010). EE pretreatment strongly enhanced resistance against *Hpa*. Remarkably, less than 10% of systemic leaves from EE-treated plants showed symptoms of infection, whereas more than 90% of control plants were infected. On the contrary, the Pip-deficient mutant *ald1* was fully infected independently of previous EE treatment (Fig. 4A). Similarly, the spore number on systemic leaves of EE-treated plants was drastically reduced in Col-0 whereas this effect was much less pronounced in *ald1* (Fig. 4B). These results illustrate a broad range protective effect of *P. brassicae* EE treatment and the important role of the NHP pathway in this response.

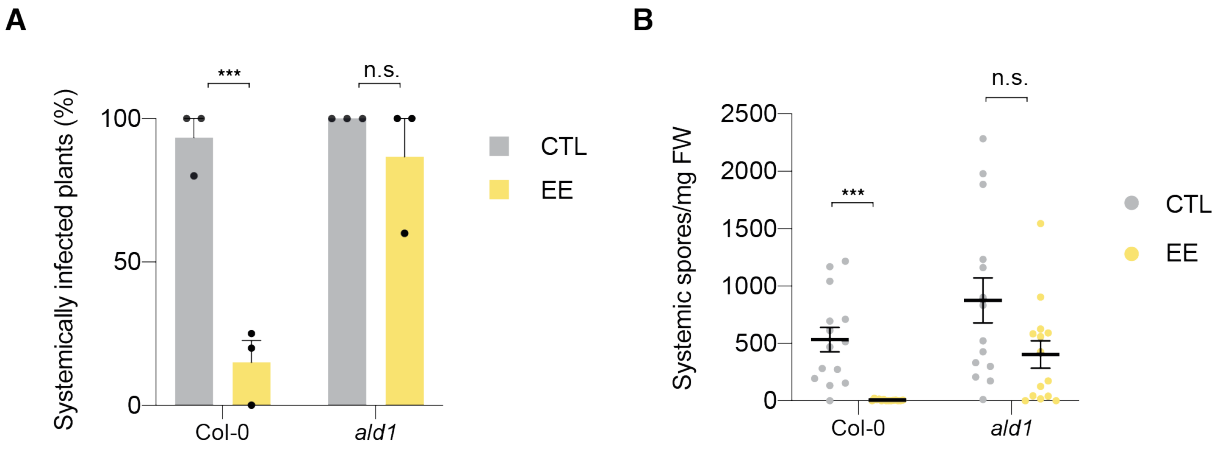


Figure 4. EE-induced SAR reduces *Hyaloperonospora arabidopsidis* infection.
Effect of one day-pretreatment with *P. brassicae* EE on *H. arabidopsidis* infection in distal leaves was measured 8 days after inoculation. Inoculated plants without pretreatment were used as controls. Percentage of systemically infected plants (**A**) or number of spores on systemic leaves (**B**) were quantitated. Mean ± SE of three independent experiments are shown (n = 4-5 *leaves per experiment*). Significant differences between control and treated plants are indicated ((**A**) Pearson's chi-squared test; (**B**) Welch's two sample *t*-test, *** P<0.001; n.s., not significant).

DISCUSSION

Egg-induced interplant SAR against *P. syringae* has been reported recently (Orlovskis and Reymond, 2020). As EE-treated *ald1* and *fmo1* mutants did not induce interplant SAR in Col-0 neighbouring plants, this suggests that the root-derived mobile signal is generated by the NHP pathway. On the contrary, SA was not required for the generation of such signal(s), as SA-deficient mutants successfully induced interplant SAR (Orlovskis and Reymond, 2020). We found here that EE treatment also induced interplant SAR against *B. cinerea*. However, whether this response uses similar signalling mechanisms remains unknown and should be further investigated. Intriguingly, interplant SAR against *P. syringae* was not induced when plants were first inoculated with the same pathogen, suggesting a specific contribution of eggs in this response, at least in the conditions tested, which suggests a beneficial role of this response for the insect (Orlovskis and Reymond, 2020). Indeed, feeding larvae are likely to spread to neighbouring plants, either to reach supplementary food or to escape host induced defences. By inducing reduction of microbial infection in surrounding plants, insects might create a niche of healthy food and thus optimise survival of hatching larvae. We demonstrated in Chapter 2 that camalexin accumulation is required for intraplant SAR against *B. cinerea*. It would be interesting to test whether this metabolite is also required for EE-induced interplant SAR establishment. Camalexin did not accumulate in distal leaves following EE treatment (Chapter

2, Fig. 3A), which suggests that its role as mobile interplant SAR signal seems unlikely. However, its *de novo* biosynthesis in neighbouring plants following perception of a root-mobile signal is possible. Treatment of Col-0 plants with EE and subsequent infection of camalexin-deficient neighbouring plants should help answering this question. Identifying the nature of the root-mediated signal in this response will also constitute an important task in the future.

P. brassicae performance was impacted when fed on *P. syringae*-infected plants (Hilfiker et al., 2014). Interestingly, *B. cinerea* infection similarly impacted *P. brassicae* performance. Whether *B. cinerea* displays direct entomopathogenic effects against *P. brassicae* is not known. *In vitro* toxicity assays of *B. cinerea* metabolites towards *P. brassicae* cell lines, as developed in (Fornelli et al., 2004) would be useful to further investigate this aspect. Alternatively, *B. cinerea* might compete with *P. brassicae* for the same resources and *Arabidopsis* might become less nutritious to larvae. By activating a SAR of broad specificity, which even extends to neighbouring plants, insects may thus increase survival of their offspring on plants potentially exposed to a variety of pathogens. It is intriguing that *Arabidopsis* triggers a SAR in response to oviposition and that this benefits the attacking insect. It is possible that during years of co-evolution, *P. brassicae* has evolved the ability to hijack the SA pathway through oviposition and activate whole-plant resistance to benefit its own progeny. In line with this, it was previously shown that egg-induced SA accumulation led to suppression of the JA pathway, therefore increasing performance of the generalist *S. littoralis* (Bruessow et al., 2010). On the other hand, other reports indicate that insect oviposition triggers the induction of anti-herbivore defence responses, therefore reducing larval performance (Bandoly et al., 2015; Geuss et al., 2018; Lortzing et al., 2019). Thus, oviposition-induced SA accumulation might have different outcomes depending on the plant/insect species combination used. Wounds caused by larval feeding form potential entry sites for pathogens and could provide an alternative explanation for egg-induced SAR. It could be a plant response that aims to reduce pathogen attacks instead of herbivory. *B. cinerea* infection might negatively affect plant fitness more than herbivory. It was reported that wounding of *Arabidopsis* leaves induced resistance to *B. cinerea* by priming camalexin accumulation (Chassot et al., 2008). It would be interesting to compare the impact of herbivory and *B. cinerea* infection on fitness of various plant species.

P. brassicae is a specialist herbivore and egg-induced SAR might be activated exclusively in relevant hosts of the *Brassicaceae* family. We indeed showed that treatments with *P. brassicae* EE induced local resistance against *B. cinerea* in four *Brassicaceae* crop species. Notably, this protection reached distal leaves only in *B. napus*. Among each plant species used, *B. napus* was the smallest of all, which could favour the systemic propagation of

EE-derived SAR signals. Although the quantity of EE applied was adjusted to the leaf size, we may have underestimated the number of eggs that could be deposited on such bigger leaves during natural oviposition or may have not waited long enough to observe a SAR. Thus, it would be crucial to repeat these experiments using natural oviposition instead of EE treatments and with extended time points. However, EE-induced resistance to *B. cinerea* was not observed in local or distal leaves of tomato plants, which suggests a family specificity of this response. It would be interesting to test whether oviposition and treatment with EE of a *Solanaceae* specialist herbivore would trigger resistance to *B. cinerea* in tomato, potato, or tobacco plants. Consistent with this hypothesis, it was recently shown that hypersensitive response (HR)-like necrosis in *B. nigra* was strongly induced following oviposition by crucifers specialist insects and only mildly induced after oviposition by non-specialist insects (Griese et al., 2021). A recent study reported that the SAR signal NHP accumulates in various plants, including cucumber, tobacco, soybean, tomato as well as barley in response to adapted pathogen infections (Schnake et al., 2020). Again, whether oviposition by specialist herbivores induces NHP accumulation and therefore SAR in these species is unknown and would be interesting to study. In addition, effect of EE treatment from various insects on a wide range of plant species might reveal interesting specificities.

Many crop species are exposed to pathogenic fungal infections, which cause important yield losses every year. To diminish these losses, chemical pesticides are widely used, but studies on their deleterious effects on the environment and human health is increasing (Beketov et al., 2013; Nicolopoulou-Stamati et al., 2016). Egg-derived phosphatidylcholines (PCs) induce immune responses in *Arabidopsis* (Stahl et al., 2020), and treatment with a PC-Mix solution induces local and systemic reduction of *B. cinerea* growth, similar to egg treatments (Chapter 1). Whether PC treatments increase resistance to fungal pathogens in crops has not been tested so far. Recently, application of bacteria-derived rhamnolipids induced foliar protection against *B. cinerea* in *B. napus* (Monnier et al., 2018). Further experiments using different crop species and various elicitors of lipidic nature could help in the development of environmental-friendly biocontrol strategies against microbial pathogens.

We showed that EE-induced SAR also targets the oomycete *Hpa* in an NHP-dependent manner. This supports recent findings where resistance to *Hpa* is enhanced following exogenous application of Pip and NHP (Hartmann et al., 2018). Whether EE-induced reduction of *Hpa* growth also relies on camalexin is unknown. However, camalexin accumulates in response to *Hpa* infection (Mert-Türk et al., 2003) although other reports have indicated that the indolic double mutant *cyp79b2 cyp79b3* and camalexin-deficient *pad3-1* mutant do not

show enhanced susceptibility, which suggests that camalexin or other tryptophan-derived metabolites are not crucial for resistance against this pathogen (Glazebrook et al., 1997; Stuttmann et al., 2011).

In conclusion, we have shown that EE-induced SAR is effective against pathogens with different lifestyles, with a conserved signalling mechanism involving the NHP pathway. EE-induced protection is also observed in important crops from the *Brassicaceae* family. Moreover, EE-induced SAR seems to benefit the insect, by increasing performance of neonate larvae. Overall, these aspects bring fascinating insights on how plants respond to a combination of different attackers and illustrate a novel facet of plant-insect interactions.

MATERIALS AND METHODS

Plant and Insect Growth Conditions

Arabidopsis thaliana (Col-0), *Brassica napus*, *Raphanus sativus*, *Brassica nigra*, *Brassica oleracea* and *Solanum lycopersicum* plants were sown in moist potting compost. After seed stratification for 2 days at 4°C, plants were grown for 4-5 weeks in growth chambers in short day conditions (10 h light/14 h dark), under 100 µmol m^{-2} s^{-1} of light, at 20-22°C and 65% relative humidity. For *Hyaloperonospora arabidopsidis* disease assays, Col-0 and *ald1-1* plants were grown on potting soil (mix z2254, Primasta B.V., Asten, The Netherlands) at 21 °C, 75% relative humidity, under short days conditions (10 h light/14 h dark).

Lines used in this study: *ald1* (Návarová et al., 2012), *B. napus* (Saatzucht Bardowick, Germany), *R. sativus* (Germline, France), *B. nigra* seeds were collected from naturally growing populations in The Netherlands (Pashalidou et al., 2015), *B. oleracea* var. *gemmifera* (Zollinger, Switzerland) and *S. lycopersicum* accession Micro-Tom (Elejalde-Palmett et al., 2021).

A population of the Large White butterfly *Pieris brassicae* was maintained on *B. oleracea* var. *gemmifera* in a greenhouse at 24°C and 65% relative humidity (Reymond et al., 2000).

Treatment with EE

For EE preparation, *P. brassicae* eggs were crushed with a pestle in Eppendorf tubes. After centrifugation (14,000 *g* for 3 min), the supernatant (EE) was collected and stored at -20°C. For application, 2 x 2 µl of EE were spotted under the surface of each of two leaves on at least 4-6 plants per independent experiment. Plants were treated 5 days before *B. cinerea* infection. Untreated plants were used as controls.

For *R. sativus* and *B. napus*, 6 x 2 µl and 3 x 2 µl, respectively, of EE were spotted under the surface of each of two leaves on at least 10 plants per independent experiment. For *B. nigra*, *B. oleracea* and *S. lycopersicum*, 6 x 2 µl of EE were spotted under the surface of each of two leaves on at least 3 plants per independent experiment.

For intra- and interplant SAR experiments, four plants were grown equidistant from each other in conical plastic pots (dia_{top} = 7 cm, dia_{base} = 5 cm, vol_{soil} = 130 ml) according to a previously published protocol (Orlovskis and Reymond, 2020). For *P. brassicae* EE treatment,

2 x 2 µl were spotted under the surface of each of three leaves on 4 plants/pot (intraplant) or 2 plants/pot (interplant). After 5 days, distal leaves from EE-treated plants, leaves from control plants grown in separate pots and leaves from untreated neighbors grown next to EE-treated plants were infected with *B. cinerea*.

Culture of *B. cinerea*, Infection and Growth Assessment

B. cinerea strain BMM (Zimmerli et al., 2001) was grown on 1X PDA (Potato Dextrose Agar, 39 g l^{-1}, Difco) for 10-14 days in darkness at 23°C. Spores were harvested in water and filtered through wool placed in a 10 ml tip to remove hyphae. Spores were diluted in half-strength PDB (Potato Dextrose Broth, 12 g l^{-1}, Difco) to a concentration of 5 x 10^5 spores ml^{-1} for inoculation.

For *Arabidopsis*, one 5 µl droplet of spore suspension was deposited on the adaxial surface of two leaves per plant. For *R. sativus*, *B. nigra* and *B. oleracea*, two leaves per plant were cut, placed on wet tissue in a tray and subsequently infected with one 20 µl droplet of spore suspension deposited on the adaxial surface. For *B. napus* and *S. lycopersicum*, the same procedure was used, except that one drop of 10 µl was deposited. Inoculated plants/leaves were kept under a water-sprayed transparent lid to maintain high humidity in a growth chamber under dim light (around 2 µmol m^{-2} s^{-1}) during the whole time of infection.

Arabidopsis were infected for 3 days before lesion measurements. *B. napus*, *R. sativus* and *S. lycopersicum* were infected for 5 days before lesion measurements. *B. nigra* and *B. oleracea* were infected for 7 days before lesion measurements. Lesion size measurements were made using ImageJ software version 2.0.0-rc-65/1.51u (http://imagej.nih.gov/ij).

Insect performance assays

Four-week-old Arabidopsis plants were sprayed with either half-strength PDB or *B. cinerea* spore suspension at a concentration of 5 x 10^5 spores ml^{-1}. After 48 h, five freshly hatched *P. brassicae* larvae were placed on each of 11 pots, each containing 2 plants. Plants were placed in a transparent plastic box and kept in a growth chamber during the experiment. Plants were replaced every 3 days by a new set of inoculated plants in order to keep a constant amount of material for feeding larvae. After 6 days of feeding, larvae were weighed on a precision balance (Mettler-Toledo, Greifensee, Switzerland) and placed back on the plants until a final weight measurement after 12 days. Each experiment was done three times (different sampling dates).

Infection with *Hyaloperonospora arabidopsidis*

Infection assays were performed with *H. arabidopsidis* isolate Noco2 (100 spores per µl). The downy mildew pathogen was maintained on *Arabidopsis* Col-0 and transferred weekly to fresh 10-day old seedlings. Spores were collected from Ws-*eds1* mutant to achieve the high level of inoculum used. Two leaves of each tested plant were treated with 2 x 2 µl of EE one day before pathogen challenge. Untreated plants were used as controls. Then, the *H. arabidopsidis* spore suspension was applied with a spray gun. Plants were subsequently left to dry to the air for ~ 30 min and incubated at 100% humidity at 16 °C under short day conditions (10 h light/ 14 h dark). Eight days post inoculation disease severity was determined visually. For spore counts, four systemic leaves from 4-5 EE-treated or control plants were cut, weighed, and suspended in 2 ml of water after which the number of spores per milligram of plant tissue was determined. Each experiment was done three times (different sampling dates).

Statistical Analyses

Statistical analyses were performed using R software version 3.5.2 (http://www.R-project.org). Normal distribution and variance homogeneity of data were evaluated with Shapiro-Wilk and Levene's test, respectively. If not normal, data were log-transformed to ensure analyses with parametric tests.

To compare CTL vs EE within the same genotype in SAR bioassays, we used a linear mixed model fit by the restricted maximum likelihood (REML) algorithm (package "lme4" in R) using plant treatment as a fixed factor and experimental block as a random factor.

For feeding bioassays, we used Welch's t-test.

AUTHOR CONTRIBUTIONS

Esteban Alfonso performed the experiments and analysed data for all the figures except for Figure 2 where bioassays using *R. sativus* and *B. napus* plants were performed and analysed by Etienne Bellani. Bioassays using *Hpa* were performed by Tom Raaymakers in the group of Guido Van den Ackerveken.

Esteban Alfonso wrote the Chapter and Philippe Reymond reviewed and edited the text.

REFERENCES

Bandoly M, Hilker M, Steppuhn A (2015) Oviposition by *Spodoptera exigua* on *Nicotiana attenuata* primes induced plant defence against larval herbivory. Plant J **83**: 661–672

Beketov MA, Kefford BJ, Schafer RB, Liess M (2013) Pesticides reduce regional biodiversity of stream invertebrates. Proceedings of the National Academy of Sciences **110**: 11039–11043

Bruessow F, Gouhier-Darimont C, Buchala A, Metraux J-P, Reymond P (2010) Insect eggs suppress plant defence against chewing herbivores: Insect eggs and plant defence. The Plant Journal **62**: 876–885

Chassot C, Buchala A, Schoonbeek H, Métraux J-P, Lamotte O (2008) Wounding of Arabidopsis leaves causes a powerful but transient protection against Botrytis infection. The Plant Journal 13

Chen Y-C, Holmes EC, Rajniak J, Kim J-G, Tang S, Fischer CR, Mudgett MB, Sattely ES (2018) *N* -hydroxy-pipecolic acid is a mobile metabolite that induces systemic disease resistance in *Arabidopsis*. Proc Natl Acad Sci USA **115**: E4920–E4929

Cheol Song G, Sim H-J, Kim S-G, Ryu C-M (2016) Root-mediated signal transmission of systemic acquired resistance against above-ground and below-ground pathogens. Ann Bot **118**: 821–831

Coates ME, Beynon JL (2010) *Hyaloperonospora arabidopsidis* as a Pathogen Model. Annu Rev Phytopathol **48**: 329–345

Dean R, Van Kan JAL, Pretorius ZA, Hammond-Kosack KE, Di Pietro A, Spanu PD, Rudd JJ, Dickman M, Kahmann R, Ellis J, et al (2012) The Top 10 fungal pathogens in molecular plant pathology: Top 10 fungal pathogens. Molecular Plant Pathology **13**: 414–430

Delory BM, Delaplace P, Fauconnier M-L, du Jardin P (2016) Root-emitted volatile organic compounds: can they mediate belowground plant-plant interactions? Plant Soil **402**: 1–26

Elejalde-Palmett C, Martinez San Segundo I, Garroum I, Charrier L, De Bellis D, Mucciolo A, Guerault A, Liu J, Zeisler-Diehl V, Aharoni A, et al (2021) ABCG transporters export cutin precursors for the formation of the plant cuticle. Current Biology **31**: 2111-2123.e9

Fornelli F, Minervini F, Mulè G (2004) CYTOTOXICITY INDUCED BY NIVALENOL, DEOXYNIVALENOL, AND FUMONISIN B 1 IN THE SF-9 INSECT CELL LINE. 6

Fu ZQ, Dong X (2013) Systemic Acquired Resistance: Turning Local Infection into Global Defense. Annu Rev Plant Biol **64**: 839–863

Geuss D, Lortzing T, Schwachtje J, Kopka J, Steppuhn A (2018) Oviposition by Spodoptera exigua on Solanum dulcamara Alters the Plant's Response to Herbivory and Impairs Larval Performance. IJMS **19**: 4008

Glazebrook J, Zook M, Mert F, Kagan I, Rogers EE, Crute IR, Holub EB, Hammerschmidt R, Ausubel FM (1997) Phytoalexin-Deficient Mutants of Arabidopsis Reveal That *PAD4* Encodes a Regulatory Factor and That Four *PAD* Genes Contribute to Downy Mildew Resistance. Genetics **146**: 381–392

Griese E, Caarls L, Bassetti N, Mohammadin S, Verbaarschot P, Bukovinszkine'Kiss G, Poelman EH, Gols R, Schranz ME, Fatouros NE (2021) Insect egg-killing: a new front on the evolutionary arms-race between brassicaceous plants and pierid butterflies. New Phytol **230**: 341–353

Hartmann M, Zeier J (2019) N-hydroxypipecolic acid and salicylic acid: a metabolic duo for systemic acquired resistance. Current Opinion in Plant Biology **50**: 44–57

Hartmann M, Zeier T, Bernsdorff F, Reichel-Deland V, Kim D, Hohmann M, Scholten N, Schuck S, Bräutigam A, Hölzel T, et al (2018) Flavin Monooxygenase-Generated N-Hydroxypipecolic Acid Is a Critical Element of Plant Systemic Immunity. Cell **173**: 456-469.e16

Hilfiker O, Groux R, Bruessow F, Kiefer K, Zeier J, Reymond P (2014) Insect eggs induce a systemic acquired resistance in Arabidopsis. Plant J **80**: 1085–1094

Little D, Gouhier-Darimont C, Bruessow F, Reymond P (2007) Oviposition by Pierid Butterflies Triggers Defense Responses in Arabidopsis. Plant Physiol **143**: 784–800

Lortzing V, Oberländer J, Lortzing T, Tohge T, Steppuhn A, Kunze R, Hilker M (2019) Insect egg deposition renders plant defence against hatching larvae more effective in a salicylic acid-dependent manner. 14

Mert-Türk F, Bennett MH, Mansfield JW, Holub EB (2003) Camalexin accumulation in Arabidopsis thaliana following abiotic elicitation or inoculation with virulent or avirulent Hyaloperonospora parasitica. Physiological and Molecular Plant Pathology **62**: 137–145

Monnier N, Furlan A, Botcazon C, Dahi A, Mongelard G, Cordelier S, Clément C, Dorey S, Sarazin C, Rippa S (2018) Rhamnolipids From Pseudomonas aeruginosa Are Elicitors Triggering Brassica napus Protection Against Botrytis cinerea Without Physiological Disorders. Front Plant Sci **9**: 1170

Návarová H, Bernsdorff F, Döring A-C, Zeier J (2012) Pipecolic Acid, an Endogenous Mediator of Defense Amplification and Priming, Is a Critical Regulator of Inducible Plant Immunity. Plant Cell **24**: 5123–5141

Nicolopoulou-Stamati P, Maipas S, Kotampasi C, Stamatis P, Hens L (2016) Chemical Pesticides and Human Health: The Urgent Need for a New Concept in Agriculture. Front Public Health. doi: 10.3389/fpubh.2016.00148

Ninkovic V, Rensing M, Dahlin I, Markovic D (2019) Who is my neighbor? Volatile cues in plant interactions. Plant Signaling & Behavior **14**: 1634993

Orlovskis Z, Reymond P (2020) *Pieris brassicae* eggs trigger interplant systemic acquired resistance against a foliar pathogen in Arabidopsis. New Phytol **228**: 1652–1661

Pashalidou FG, Fatouros NE, Van Loon JJA, Dicke M, Gols R (2015) Plant-mediated effects of butterfly egg deposition on subsequent caterpillar and pupal development, across different species of wild Brassicaceae: Butterfly egg deposition on subsequent herbivory. Ecol Entomol **40**: 444–450

Reymond P, Weber H, Damond M, Farmer EE (2000) Differential Gene Expression in Response to Mechanical Wounding and Insect Feeding in Arabidopsis. 14

Riedlmeier M, Ghirardo A, Wenig M, Knappe C, Koch K, Georgii E, Dey S, Parker JE, Schnitzler J-P, Vlot AC (2017) Monoterpenes Support Systemic Acquired Resistance within and between Plants. Plant Cell **29**: 1440–1459

Schnake A, Hartmann M, Schreiber S, Malik J, Brahmann L, Yildiz I, von Dahlen J, Rose LE, Schaffrath U, Zeier J (2020) Inducible biosynthesis and immune function of the systemic acquired resistance inducer N-hydroxypipecolic acid in monocotyledonous and dicotyledonous plants. Journal of Experimental Botany **71**: 6444–6459

Stahl E, Brillatz T, Ferreira Queiroz E, Marcourt L, Schmiesing A, Hilfiker O, Riezman I, Riezman H, Wolfender J-L, Reymond P (2020) Phosphatidylcholines from Pieris brassicae eggs activate an immune response in Arabidopsis. eLife **9**: e60293

Sticher L, Mauch-Mani B, Métraux J-P (1997) SYSTEMIC ACQUIRED RESISTANCE. 36

Stout MJ, Thaler JS, Thomma BPHJ (2006) PLANT-MEDIATED INTERACTIONS BETWEEN PATHOGENIC MICROORGANISMS AND HERBIVOROUS ARTHROPODS. Annu Rev Entomol **51**: 663–689

Stuttmann J, Hubberten H-M, Rietz S, Kaur J, Muskett P, Guerois R, Bednarek P, Hoefgen R, Parker JE (2011) Perturbation of *Arabidopsis* Amino Acid Metabolism Causes Incompatibility with the Adapted Biotrophic Pathogen *Hyaloperonospora arabidopsidis*. Plant Cell **23**: 2788–2803

Wenig M, Ghirardo A, Sales JH, Pabst E, Breitenbach H, Antritter F, Weber B, Lange B, Lenk M, Cameron R, et al (2019) Systemic acquired resistance networks amplify airborne defense cues. 14

Williamson B, Tudzynski B, Tudzynski P, Van Kan JAL (2007) Botrytis cinerea: the cause of grey mould disease. Mol Plant Pathol **8**: 561–580

Zimmerli L, Métraux J-P, Mauch-Mani B (2001) β-Aminobutyric Acid-Induced Protection of Arabidopsis against the Necrotrophic Fungus Botrytis cinerea. Plant Physiology **126**: 517–523

GENERAL DISCUSSION

Egg-induced SAR against *Botrytis cinerea*

Pieris brassicae herbivory activates defence responses regulated by the JA pathway. Remarkably, oviposition by the same species triggers defences modulated by the SA pathway, which has been shown to act antagonistically to the JA pathway (Pieterse et al., 2012). Experiments conducted in our laboratory demonstrated that egg-induced SA accumulation can inhibit JA-dependent defences, for the benefice of hatching larvae, which displayed increased performance in oviposited plants (Bruessow et al., 2010). In this thesis, we focused on another egg-induced SA response, which is the activation of SAR. It was previously shown that *P. brassicae* oviposition and egg extract (EE) treatment reduce growth of several strains of the bacterial pathogen *Pseudomonas syringae* through the activation of SAR in *Arabidopsis* (Hilfiker et al., 2014). The aim of this thesis was to determine whether egg-induced SAR was also effective against the fungal pathogen *Botrytis cinerea*. We showed that *P. brassicae* oviposition and EE treatment efficiently reduce growth of this pathogen. In addition, we further characterised the signalling mechanisms and determined a functional role of the tryptophan-derived indolic metabolism in egg-induced SAR establishment. However, although these findings bring interesting perspectives, they also raise several questions.

By using mutants impacted in various branches of the indolic metabolism, we identified the phytoalexin camalexin as being a crucial component for EE-induced SAR against *B. cinerea*. Although several camalexin-deficient mutants are impacted in EE-induced SAR, which genetically supports a role of camalexin in this response, chemical analyses did not reveal significant differences in camalexin levels in infected plants previously treated with EE or not. Indeed, a logical explanation for the camalexin-driven reduction of *B. cinerea* growth would have been to detect primed accumulation of this metabolite in infected leaves of EE-treated plants, which we did not. The question is thus how EE treatment reduces *B. cinerea* growth through camalexin? As discussed in Chapter 2, fungal pathogens can actively detoxify or export antifungal compounds. It has been shown that *B. cinerea* detoxifies camalexin into indole-3-acetonitrile and indole-3-carboxylic acid, which are less antifungal (Pedras et al., 2011). One possibility would be that EE pretreatment inhibits camalexin detoxification in *B. cinerea*. This would explain the similar accumulation of camalexin in control and EE-treated plants and this mechanism would target *B. cinerea* metabolism. Identification of *B. cinerea* enzymes involved

in camalexin catabolism would be necessary for further monitoring of their expression/activation after infection of EE-treated plants. However, what signals from EE-treated plants would be involved and how they would be delivered to *B. cinerea* is unknown. It has been recently shown that during *B. cinerea* infection, *Arabidopsis* sends extracellular vesicles containing small RNAs targeting *B. cinerea* virulence genes for silencing (Cai et al., 2018). One hypothesis could be that small RNAs are generated and delivered to the infection site through extracellular vesicles following EE treatment. These small RNAs could potentially silence *B. cinerea* genes involved in camalexin detoxification. Further work will be required to investigate this hypothesis. Alternatively, camalexin might be metabolised *in planta* to yet unidentified metabolites with antifungal activity and EE pretreatment would enhance this conversion. The synthesis of radio-labelled camalexin and its use in camalexin-deficient mutants would allow to follow its metabolism and identify possible catabolites.

SA and NHP metabolically act together to orchestrate SAR (Hartmann and Zeier, 2019). Consequently, the absence of one or the other ultimately affects SAR establishment in plants, although a minor SA-independent SAR can be activated in a NHP-dependent manner (Bernsdorff et al., 2016). We confirmed in Chapter 1 that mutants impacted in SA and NHP biosynthesis do not activate EE-induced SAR. In addition, we showed that EE-induced SAR required functional SA signalling, since *npr1-1* and *npr1-1 npr4-4D* mutants showed severe or full inhibition of EE-induced SAR activation, respectively. As discussed, we link EE-induced SAR establishment with camalexin accumulation. However, we found that *ald1* and *ics1* mutants, which are impacted in NHP and SA biosynthesis, respectively, accumulate wild-type levels of camalexin although they are impaired in EE-induced SAR. Furthermore, *npr1-1 npr4-4D*, which is fully SA-insensitive, was more susceptible to *B. cinerea*. These results raise the question of whether NPR SA receptors regulate camalexin biosynthesis, which has not been demonstrated so far. Quantification of camalexin in response to EE treatment and/or *B. cinerea* infection in *npr1-1 npr4-4D* will be necessary to answer this question. A recent report indicated that NHP watering primes plants through NPR1 for enhanced defence activation, including SA biosynthesis, camalexin accumulation and defence gene expression (Yildiz et al., 2021). Whether NHP binds to NPR1 in distal leaves to elicit these responses remains unknown and future work should address this point. Overall, these results show that SA and NHP pathways are required in local leaves for the generation and transmission of SAR signal(s).

SAR is activated in response to pathogen infection or chemical treatment occurring in the local leaf, which constitutes the site of primary inoculation. Resistance is then induced locally, but also spreads in distal leaves of the plant to prevent secondary infection (Fu and

Dong, 2013). In this study, we showed that natural oviposition or EE treatment in local leaves increased resistance to *B. cinerea* in distal leaves. By using mutants impacted in various pathways, we identified several actors involved in this response. However, it is sometimes difficult to precisely affirm the site of their action. For example, we showed that *ics1*, *ald1* and *fmo1* are impaired in EE-induced SAR and postulated that the SA and NHP pathways are required in local leaves for the generation and transmission of SAR signals. However, whether ICS1, ALD1 and FMO1 enzymes are also required in distal leaves for SAR establishment cannot be discriminated by using such mutants. Similarly, we postulated that the indolic metabolism is required for the reduction of *B. cinerea* growth in distal leaves, but again, whether this pathway plays a role in local leaves cannot be excluded. Initial experiments investigating the nature of the transmitted SAR signal used grafting (Gaffney et al., 1993; Vernooij et al., 1994). The use of leaf grafting in *Arabidopsis* would be useful to generate chimeras of different genotypes to better study the site of action of each actor in EE-induced SAR. However, grafting techniques are mainly used to combine rootstocks and scions at the hypocotyl region, which represents a thicker region compared to leaf petioles and thus more suitable for such delicate manipulations. One alternative could be the generation of transgenic lines expressing genes of interest under the control of an inducible promoter. For example, a line expressing *NPR1* and *NPR4* under dexamethasone (DEX)-inducible promoters would be useful to investigate whether SA signalling is required in local or distal leaves, or in both. One could spray DEX specifically in local EE-treated leaves and infect distal leaves with *B. cinerea* or on the contrary spray distal leaves before infection and observe whether EE-induced SAR could be restored in such line.

Defences against *B. cinerea* are usually known to require JA/ET pathways (Pieterse et al., 2012). However, the observation that mutants blocked in SA signalling are more susceptible to this pathogen invokes a role for SA in defence against *B. cinerea*. During the early phase of infection, *B. cinerea* silences host defence genes by the translocation of small RNAs (Weiberg et al., 2013). This requires the host plant to be alive and therefore suggests that *B. cinerea* has a short biotrophic phase before triggering host cell death. This phase should last few hours but is crucial for *B. cinerea* infection success. During this phase, SA might be activated, and this would explain the enhanced susceptibility of several mutants impacted in the SA pathway. It would be interesting to measure SA levels in the early phase of the infection with the strain used in our study, to check whether it correlates with the increased susceptibility of SA signalling mutants.

Although several steps remain unclear, we propose a model depicting the main actors of EE-induced SAR against *B. cinerea* (Fig. 1). *Arabidopsis* locally detects egg-derived phosphatidylcholines (PCs), possibly through the lectin receptor kinase LecRK-I.8 (Gouhier-Darimont et al., 2019; Stahl et al., 2020). This triggers phosphorylation of MPK3 and MPK6 (C. Gouhier-Darimont, unpublished) and the activation of SA and NHP pathways (Hilfiker et al., 2014). NHP is the metabolic regulator of SAR and can move systemically, where it boosts SA production and defence gene expression (Zeier, 2021). Whether it has a direct function on camalexin biosynthesis remains unclear. In distal leaves, *B. cinerea* infection induces camalexin biosynthesis, which is regulated by WRKY33. As previously discussed, it remains unclear how egg-induced responses inhibit *B. cinerea* through camalexin, since no primed accumulation of this metabolite was measured in distal leaves of EE-treated plants.

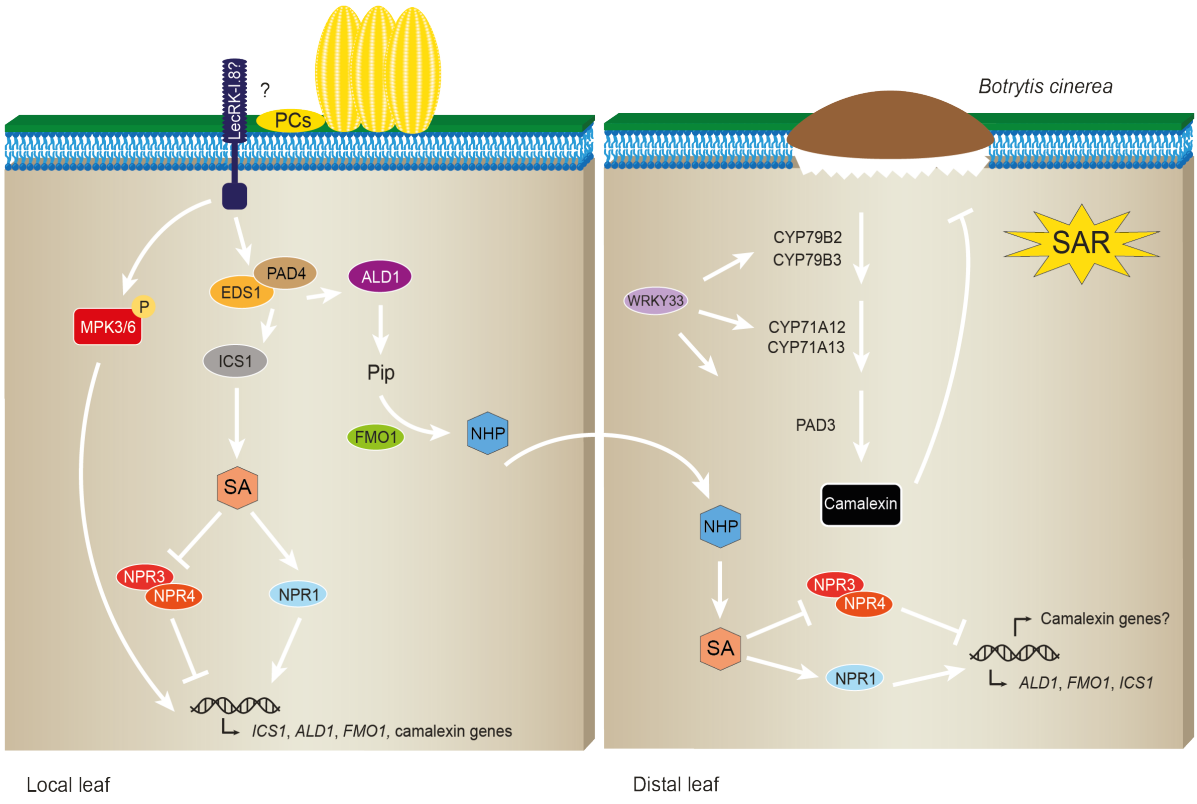


Figure 1. Simplified model of egg-induced SAR against *B. cinerea*.
Refer to the text for details.

We also observed a substantial accumulation of indole-3-carbaldehyde (ICHO) and indole-3-carboxylic acid (ICA) glucose conjugates in systemic leaves of EE-treated plants (Chapter 3). We showed that this accumulation was abolished in *ald1* mutant, and that Pip watering did not restore this phenotype, suggesting that ALD1 enzyme and not Pip, its enzymatic product, is responsible for such accumulation. Accumulation of these conjugates was also completely abolished in the *myb34 myb51 myb122* triple mutant (*tmyb*), devoid of indole glucosinolates. Intriguingly, *ald1* and *tmyb* mutants accumulated wild-type levels of the precursors ICHO and ICA, suggesting that only the glycosylation is affected. We propose a speculative model of how ALD1 and MYB34/51/122 contribute to the formation of these ICHO/ICA conjugates (Fig. 2). Treatment of local leaves with EE induces the expression of *ALD1* (Hilfiker et al., 2014). We showed that the expression of several genes coding for UDP-glycosyltransferases (UGTs) was inhibited in local and distal leaves of *ald1*. Whether ALD1 has a direct function on the activity of UGTs is unknown and probably unlikely, given that *ALD1* codes for an aminotransferase (Song et al., 2004). However, a study reported that ALD1 can generate non-Pip metabolites able to induce defence responses (Cecchini et al., 2015). We hypothesise that an ALD1-derived metabolite potentially regulates the expression of UGTs capable of glycosylation on ICHO and ICA. This reaction could establish in local leaves and ICHO/ICA conjugates could be transported to systemic leaves. Alternatively, the ALD1-derived metabolite could travel in systemic leaves in response to EE treatment, activate UGTs, thus generating ICHO/ICA conjugates in systemic leaves. Finally, MYB34/51/122 might have a role in direct regulation of the same UGTs in systemic leaves or ICHO/ICA conjugates might derive from yet unknown indolic metabolites directly regulated by MYB34/51/122 (Fig.2). Future work should aim at characterising these UGTs and monitor their expression in *ald1* and *tmyb* mutants. However, we showed that ICHO/ICA conjugates accumulation constitutes a consequence of EE-induced SAR and is not the cause of *B. cinerea* growth reduction, although we cannot exclude that they might be active against other pathogens.

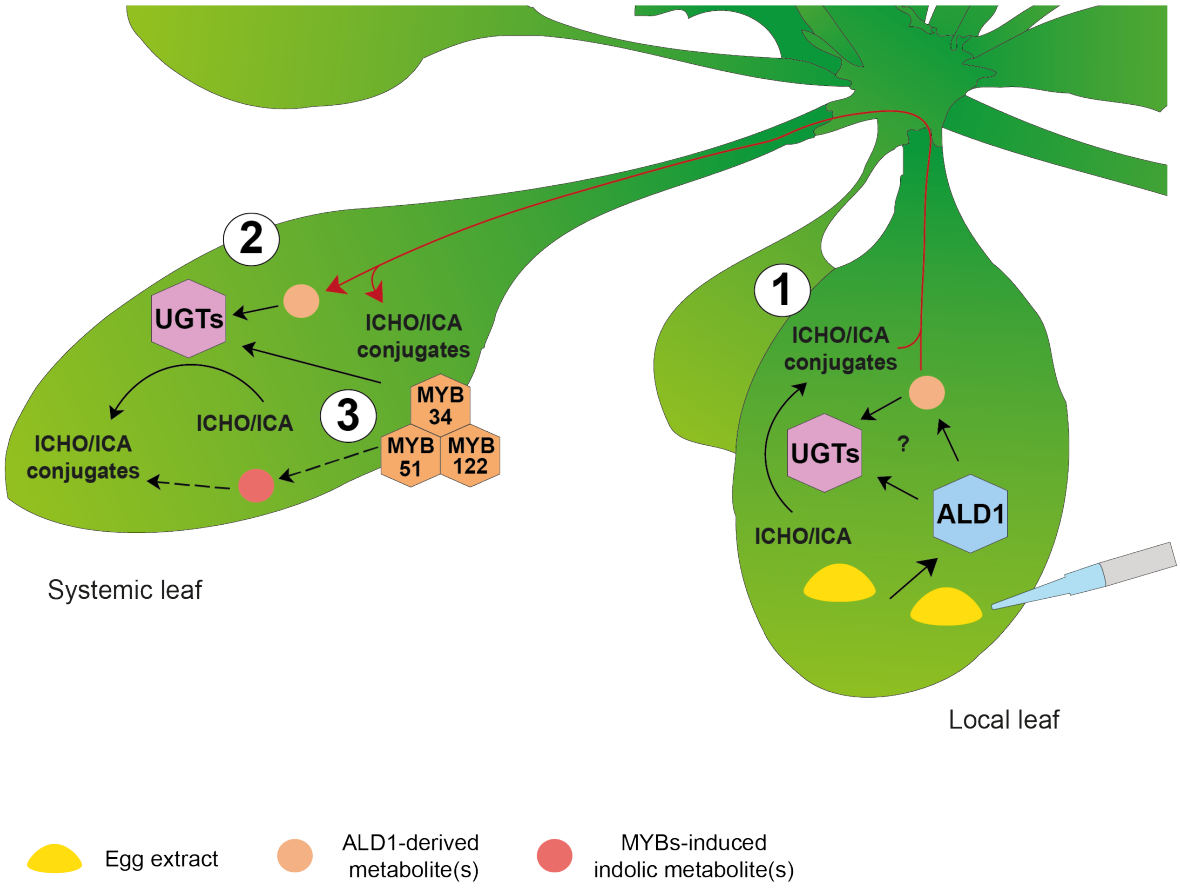


Figure 2. Speculative model showing EE-induced systemic accumulation of ICHO/ICA conjugates. Refer to the text for details.

Ecological aspects and perspectives around egg-induced SAR

Egg-induced SAR is efficient against *P. syringae*, *B. cinerea* and *Hyaloperonospora arabidopsidis*, which are pathogens with different lifestyles (Hilfiker et al., 2014; this thesis). These responses are dependent on SA and require the activation of the NHP pathway. Whether NHP-dependent egg-induced SAR can extend to other pathogens remains to be studied. Future work should aim at conducting experiments with a large variety of different pathogens to check the generality of egg-induced SAR. Also, whether egg-induced SAR can protect plants against multiple infections at once also needs further consideration. One could imagine a unique treatment efficiently reducing infection of several pathogens. We also showed that EE-induced SAR extends to untreated neighbouring plants. However, neighbouring plants used in our experiments were from the same species. It would be interesting to investigate whether an egg-

treated plant is able to transmit protection to a neighbour from another species. Whether such interspecies SAR would be restricted to the same family or not remains an open question.

P. brassicae larvae performed worse on *P. syringae*- and *B. cinerea*-infected plants (Hilfiker et al., 2014; this study). As discussed in Chapter 4, this suggests that egg-induced reduction of pathogen infection might benefit hatching larvae. One intriguing question is why the plant induces such a response and why it was conserved through evolution? Plants might face a trade-off between being infected by pathogens or eaten by herbivorous insects. It might be beneficial for plants to induce resistance against pathogens before larvae hatch and start feeding on leaf tissue. Indeed, open wounds created by larval feeding might increase pathogen entry and be more detrimental to the plant than herbivory. In this scenario, both plants and insects might benefit from egg-induced SAR. However, in an environment where plants density is scarce, larvae might be able to feed on the entire plant. Such tripartite interactions have sometimes opposite outcomes. Indeed, several studies demonstrated that some insect species performed better on plants previously infected by pathogens, and this was associated with hormonal crosstalk effects (van Mölken et al., 2014; Vos et al., 2015). Field experiments with monitoring of egg-induced SAR costs and benefits for fitness of both plants and insects would be interesting to conduct and should give a more realistic idea of what happens in natural environments. The choice of plant/insect species combination used would also need to be determined carefully.

Another point that should be addressed is how long egg-induced protection can last. Is the protective status of plants efficient against further infections several days or weeks later? Is the protective status of EE-treated plants transmitted to the next generation? Parental plants infected with *P. syringae* produced progeny that were more resistant to a first infection with *H. arabidopsidis* and *P. syringae* compared to progeny from mock-inoculated parents. Furthermore, this protective status was conserved over one stress-free generation, which indicates an epigenetic basis of this response (Luna et al., 2012). Whether EE application to other parts of the plant induces similar protection towards pathogens has not been tested. It would be interesting to compare EE treatment on different parts of a plant for subsequent leaf protection. Treatment of seeds with priming agents is extensively used and has proved to be efficient to improve germination and stress resistance of young seedlings (Paparella et al., 2015). To investigate a potential seed priming effect of EE, it would be interesting to measure pathogen resistance of plants whose seeds were treated with EE. If efficient, this would bring an alternative to the use of agrichemicals. The recent discovery that egg-derived PCs induce immune responses in *Arabidopsis* opens new horizons towards applications in agriculture

(Stahl et al., 2020). However, a multitude of tests remains to be performed, using crops in greenhouse and field experiments to assess the range and timescale of protection, as well as the impact of such treatments on yields. Alternatively, PC-induced SA accumulation might efficiently reduce pathogen infections but also impact JA levels in crop species and whether this side effect increases insect performance will have to be studied carefully.

In conclusion, our study sheds light on a novel aspect of plant-insect interactions and illustrates the complexity of the on-going arms race implicating different developmental stages of the insect and complex signalling events in *Arabidopsis*. Although the generality of these findings needs to be extended to more plant species and pathogens, they open perspectives for the development of new biocontrol methods that could be used in organic farming.

REFERENCES

Bernsdorff F, Döring A-C, Gruner K, Schuck S, Bräutigam A, Zeier J (2016) Pipecolic Acid Orchestrates Plant Systemic Acquired Resistance and Defense Priming via Salicylic Acid-Dependent and -Independent Pathways. Plant Cell **28**: 102–129

Bruessow F, Gouhier-Darimont C, Buchala A, Metraux J-P, Reymond P (2010) Insect eggs suppress plant defence against chewing herbivores: Insect eggs and plant defence. The Plant Journal **62**: 876–885

Cai Q, Qiao L, Wang M, He B, Lin F-M, Palmquist J, Huang S-D, Jin H (2018) Plants send small RNAs in extracellular vesicles to fungal pathogen to silence virulence genes. Science **360**: 1126–1129

Cecchini NM, Jung HW, Engle NL, Tschaplinski TJ, Greenberg JT (2015) ALD1 Regulates Basal Immune Components and Early Inducible Defense Responses in *Arabidopsis*. MPMI **28**: 455–466

Fu ZQ, Dong X (2013) Systemic Acquired Resistance: Turning Local Infection into Global Defense. Annu Rev Plant Biol **64**: 839–863

Gaffney T, Friedrich L, Vernooij B, Negrotto D, Nye G, Uknes S, Ward E, Kessmann H, Ryals J (1993) Requirement of Salicylic Acid for the Induction of Systemic Acquired Resistance. Science **261**: 754–756

Gouhier-Darimont C, Stahl E, Glauser G, Reymond P (2019) The Arabidopsis Lectin Receptor Kinase LecRK-I.8 Is Involved in Insect Egg Perception. Front Plant Sci **10**: 623

Hartmann M, Zeier J (2019) N-hydroxypipecolic acid and salicylic acid: a metabolic duo for systemic acquired resistance. Current Opinion in Plant Biology **50**: 44–57

Hilfiker O, Groux R, Bruessow F, Kiefer K, Zeier J, Reymond P (2014) Insect eggs induce a systemic acquired resistance in Arabidopsis. Plant J **80**: 1085–1094

Luna E, Bruce TJA, Roberts MR, Flors V, Ton J (2012) Next-Generation Systemic Acquired Resistance. Plant Physiol **158**: 844–853

van Mölken T, Kuzina V, Munk KR, Olsen CE, Sundelin T, van Dam NM, Hauser TP (2014) Consequences of combined herbivore feeding and pathogen infection for fitness of Barbarea vulgaris plants. Oecologia **175**: 589–600

Paparella S, Araújo SS, Rossi G, Wijayasinghe M, Carbonera D, Balestrazzi A (2015) Seed priming: state of the art and new perspectives. Plant Cell Rep **34**: 1281–1293

Pedras MSC, Hossain S, Snitynsky RB (2011) Detoxification of cruciferous phytoalexins in Botrytis cinerea: Spontaneous dimerization of a camalexin metabolite. Phytochemistry **72**: 199–206

Pieterse CMJ, Van der Does D, Zamioudis C, Leon-Reyes A, Van Wees SCM (2012) Hormonal Modulation of Plant Immunity. Annu Rev Cell Dev Biol **28**: 489–521

Song JT, Lu H, Greenberg JT (2004) Divergent Roles in *Arabidopsis thaliana* Development and Defense of Two Homologous Genes, *ABERRANT GROWTH AND DEATH2* and *AGD2-LIKE DEFENSE RESPONSE PROTEIN1* , Encoding Novel Aminotransferases. Plant Cell **16**: 353–366

Stahl E, Brillatz T, Ferreira Queiroz E, Marcourt L, Schmiesing A, Hilfiker O, Riezman I, Riezman H, Wolfender J-L, Reymond P (2020) Phosphatidylcholines from Pieris brassicae eggs activate an immune response in Arabidopsis. eLife **9**: e60293

Vernooij B, Reist LFM, KolditzJawhar R, Ward E, Uknes S, Kessmann H, Ryals J (1994) Salicylic Acid 1s Not the Translocated Signal Responsible for lnducing Systemic Acquired Resistance but 1s Required in Signal Transduction. 8

Vos IA, Moritz L, Pieterse CMJ, Van Wees SCM (2015) Impact of hormonal crosstalk on plant resistance and fitness under multi-attacker conditions. Front Plant Sci. doi: 10.3389/fpls.2015.00639

Weiberg A, Wang M, Lin F-M, Zhao H, Zhang Z, Kaloshian I, Huang H-D, Jin H (2013) Fungal Small RNAs Suppress Plant Immunity by Hijacking Host RNA Interference Pathways. Science **342**: 118–123

Yildiz I, Mantz M, Hartmann M, Zeier T, Kessel J, Thurow C, Gatz C, Petzsch P, Köhrer K, Zeier J (2021) The mobile SAR signal N-hydroxypipecolic acid induces NPR1-dependent transcriptional reprogramming and immune priming. Plant Physiology kiab166

Zeier J (2021) Metabolic regulation of systemic acquired resistance. Current Opinion in Plant Biology **62**: 102050

ACKNOWLEDGEMENTS

First, I would like to express my gratitude to my thesis director, Philippe, for all these years of supervision. I still remember your class during my 3rd year of Bachelor and my reaction was "Damn, this is crazy!", I knew since then I wanted to join the lab for my Master project, in which I finally stayed 5 more years... Thank you for all your support, advice, kindness and for ruling the lab your way, the atmosphere in it is amazing and you are the first responsible for that!

Philippe is gifted to always hire really nice people. Amazing people, who became real friends, I am very glad to have been part of that team for 6 years! Caroline, the second boss of the lab, thank you for your kindness and all your advice and help when I was lost in the lab! Although you rarely saw me in the morning, I really enjoyed sharing the lab with you! Raphaël, I do not even know what to say, so many memories with you, (almost) all of them crazy and limitless (sometimes too much... but we love it). Thank you for being you! Elia, thank you so much for everything, it was so good to have such a competent and crazy post-doc all along my PhD! You always said you would steal my project, but in the end your wise advice made us work together (in between few beers) and I am very grateful for this! Fabian, attaboy! It was a real pleasure for me that you joined back the lab, always funny to see you grating carrots in the lab or taking care of your snail colony! Zigmunds, thank you for the motivational discussions we had sometimes! It was nice to join you to hikes in our beautiful country! Angel, a bit more than one year that you are in the lab and yet, it feels like way longer! Always motivated for beers (and more), I really spent nice moments with you! Many more to come anyway... Maria, you joined this lab of crazy dudes, but I have the feeling you are even crazier... I will miss these little moments of gossiping! Marion, the ultimate PhD of the Reymond lab, please, do not use your martial arts skills to beat the others when they annoy you! I wish you guys all the best for the next years of your PhDs! Of course, I am very grateful for the good moments shared with the lab alumni; Olivier, my Master supervisor who initiates me to this project and Steve, my handsome boy who helped me several time!

Also, this department really has something special. I never worked in another one, but I am sure this is quite unique! A massive thank you to all past and present members for amazing parties/activities/trips/hikes, I will not forget these moments!

More serious, I am very grateful to Pauline and Elia (in addition to Philippe), who took time to read parts of this thesis and for their useful comments!

Many thanks also to my family, who never really understood what I was doing but who always supported me no matter what! Big thanks also to my other family, ma boys from MGM, for quality moments in the everyday life for 15 years now!

Many thanks to the members of my PhD committee, Paul Franken, Anke Steppuhn and Brigitte Mauch-Mani for reading this work and discussing it with me during the private defense.